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INTERNAL SECRETIONS AND TOXEMIA OF LATE PREGNANCY

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CRITICAL, COMPREHENSIVE PUBLICATIONS concerning the toxemias of pregnancy have been written by Berkeley (15), Holland (76), Hinselmann (73), Stander (174), Herrmann (71), Kosmak (83), McIlroy (102), Dieckmann (44), Dexter and Weiss (43) and Browne (26). These authors have made exhaustive studies of the literature on this subject and included their own investigations. The present review was prompted by the increased knowledge of internal secretions in relation to this diseased state, chiefly as regards the hormones elaborated by the placenta. Unfortunately, detailed morphologic studies of the glands of internal secretion in toxemia as well as in normal pregnancy are lacking (43), with the exception of the placenta and only recently has this organ been receiving more thorough histologic attention (*v.i.* under Placental Hormones).

Anterior lobe of the hypophysis. Gonadotropic hormones. No follicular activity was found in the ovaries of hypophysectomized rats following the injection of sera from patients with toxemia (5, 140). The effect noted was the same as that produced both by sera from normally pregnant women and by an extract of human placenta (140). The results supported Philipp's (112) demonstration that the hypophyses of pregnant women are gonadotropically inactive, so far as the ovaries of rodents are concerned, and indicated further that patients with toxemia do not differ in this respect from normal. The anatomical studies reviewed by Dieckmann (44) did not suggest that the anterior lobe is primarily at fault in eclampsia.

Thyrotropic hormone. Bonilla and Kramann (19) extracted a substance with properties similar to those of the thyrotropic hormone from the urine of patients with eclampsia. They did not obtain it from the urine of normal pregnant women.

Adrenotropic hormones. Fauvet and Münzner (58) reported subnormal corticotropic hormone levels in seven of eight women with severe toxemia.

Posterior lobe of the hypophysis. From a perusal of the literature and their own studies, Dieckmann (44), Dexter and Weiss (43) and Browne (26) concluded that the secretions of this gland are not implicated etiologically in pre-eclampsia

and eclampsia. Their conclusion holds for pressor as well as antidiuretic activity. Krieger and Kilvington (85) have more recently surveyed the conflicting literature concerning the antidiuretic property of blood and toxemia and tested the blood of 303 women. They found blood antidiuretic activity in 29 to 41 per cent of the groups of patients studied and that the activity in both normal and toxemic cases was related to labor and the early puerperium. Therefore they suggested that the contradictory results in the literature might be explained if considered in relation to the onset of labor.

Antidiuretic substance in the urine. Concentrates prepared from the urine of patients with toxemia were discovered to exert a marked antidiuretic effect which was not obtained with similar extracts from normal women in late pregnancy (18, 43, 67, 84, 125, 183). According to some authors, this effect was not due to pitressin (67, 125). Furthermore, a principle resembling the urinary substance was found in larger amounts in the placentas of toxemic women than in those of normal pregnancy (67).

Melanophore-expanding principle. Küstner and Dietel (87) reported a substance with this property in the blood and placentas of patients with eclampsia. Ehrhardt (51) confirmed this. Others have demonstrated this effect in the blood of toxemic women (6, 104).

Effects of administered posterior pituitary extracts. Chipman (192), Dieckmann and Michel (45) and Hofbauer (74) stated that the use of pituitary extracts in toxemia occasionally precipitates convulsions; Stander (174), referring to reports in which eclampsia appeared to ensue or become aggravated upon the injection of pituitrin, was of the opinion that the routine use of pituitary extracts in many clinics, without appreciably increased incidence of eclampsia, indicated that such observations were coincidental. In this connection, a number of patients with diabetes insipidus have been treated throughout pregnancy with posterior-lobe extracts (44, 121, 138) without any alarming developments. The following is quoted from Dexter and Weiss (43): "Because patients with diabetes insipidus are especially sensitive to the antidiuretic effect of the posterior pituitary hormone, it is worth noting here that usually such patients show no striking improvement during pregnancy and that there have even been instances in which diabetes insipidus appeared during pregnancy and disappeared after delivery."

Dieckmann and Michel (45), however, and others (25, 88, 104, 128, 187, 208) demonstrated an increased susceptibility of toxemic individuals to posterior pituitary extracts, a much higher and longer-lasting rise of blood pressure resulting. Moreover, Browne (25), using the cold pressor test and injecting tonephrin (pitressin), noted that the hypersensitivity of pre-eclamptic women to this hormone persists during the puerperium, being found as late as the two hundred thirty-fifth day. He also noted a similar cold pressor test hypersensitivity to pitressin in normal puerperal women as late as the twentieth day. From further studies he concluded (26) that the high reaction to pressor substances in pre-eclamptic toxemia is not due to predisposition but to something acquired during pregnancy and seemingly after the third month.

Inactivating power of the blood of pregnant women upon pitocin and pitressin.

Fekete and others (59, 195–197) found that posterior-lobe hormones were inactivated when mixed with serum from pregnant women. Woodbury *et al.* (208) confirmed this and noted, moreover, that the hypersensitivity of patients with toxemia to these hormones (pitressin and pitocin) was not associated with a diminished ability of their blood to inactivate them. Plasma pitocinase studies were made by Page (105). After establishing a curve of values through normal pregnancy, he found the enzyme within normal range in only 3 of 16 women with toxemia; in 7 the values were high, in 6, low. There was no correlation between the amount of enzyme and the severity of the disease.

THE THYROID GLAND. Anselmino and Hoffmann (75) presented evidence which they interpreted as demonstrating increased thyroid hormone, or a material with the same action, in the blood in normal pregnancy, the amount being enormously increased in eclampsia. No toxemia occurred in Davis' (41) eight patients with hyperthyroidism complicated by pregnancy, but five of Kibel's (82) nine similar cases developed pre-eclampsia.

It has been suggested that hypothyroidism may play a part in the production of toxemia of pregnancy (37, 38, 78, 109). Since, according to Dexter and Weiss (43), the clinical picture does not indicate hypothyroidism, the basal metabolic rate in this condition is not at myxedematous levels, and the protein content of the edema fluid is extremely low, hypofunction of the thyroid gland cannot play a significant rôle in its causation.

THE PARATHYROID GLANDS. "Many studies have been made of serum calcium in toxemic patients, but the consensus of opinion is that there are no significant changes in the total calcium or in any of its fractions in eclampsia or any of the other toxemias" (44). Patients with eclampsia and pre-eclampsia have been treated with parathyroid extracts (23, 24, 91) but without results sufficiently impressive to indicate any specificity of the therapy.

PANCREAS. *Diabetes.* The incidence of toxemia is greatly increased in diabetic patients (17, 44, 61, 63, 89, 199, 204). No one has elucidated the significance of this.

THE ADRENAL GLANDS. Fauvet and Münzer (58) found the adrenals small in six women who died of eclampsia, the weight of the two glands being less than 10.0 grams in all. In normal pregnancy the adrenal cortex is markedly increased, due to an increase in size and in fat content of the cells of the zona fasciculata, so that the adrenals are larger than in non-pregnant individuals, in whom these glands weigh 12 to 15.5 grams (8).

The adrenal medulla. The injection of epinephrin into patients with toxemia was found to cause a reversal of the usual blood pressure response, *viz.*, a drop instead of a rise (33, 92). Macchiarulo (95) recorded that an excess of epinephrin in the blood was usually present in eclamptic women and to this he attributed in part convulsions, vascular contractions and hypertension.

The adrenal cortex. The results of research on the adrenal have been reviewed by Swingle and Remington (176) but little has been done on any possible relation between adrenal function and toxemia of pregnancy. Over-activity of the adrenals has been considered (26, 43, 181). Taylor *et al.* (181) found in two

cases of toxemia after delivery "a pronounced divergence of the sodium and potassium lines which might indicate a return to normal from a previous condition of relatively low potassium and high sodium storage." "This could on theoretical grounds have been produced by hyperactivity of the adrenal cortex." Against this view, however, they pointed out their observation of potassium as well as sodium retention in toxemia, whereas lowered serum potassium and a high excretion rate are characteristic of adrenal hyperactivity. Interesting in this connection are reports concerning two patients with Addison's disease complicated by pregnancy (127, 211) who went through essentially uneventful gestations while under treatment with sodium chloride and cortical extract.

Although urinary corticoids and ketosteroids have been determined during normal pregnancy (188, which includes references to the work of others on ketosteroid excretion in pregnancy), they have not been studied in relation to toxemia.

THE OVARIES. Toxemia has not been stated to have occurred in patients whose pregnancies continued after the early removal of the corpus luteum or of both ovaries (2, 4, 8, 22, 28, 44, 49, 50, 79, 118, 123, 167, 177, 191). This may not be interpreted to mean that toxemia has never developed in such patients. On the other hand, since a fairly large number of such cases has been reported, it may be significant.

In presenting their case of eclampsia associated with ovarian pregnancy, Pride and Rucker (117) stated that it was the seventh recorded instance of toxemia in patients with pregnancies outside of the uterus.

THE PLACENTA. For decades authors have ascribed toxemia of pregnancy to abnormal placental function. The literature concerning this organ has been thoroughly covered by the writers referred to in the first paragraph of this review. Knowledge of the placental hormones, however, was only beginning to be acquired during the period they reviewed. The following statements made by Dexter and Weiss (43) summarize their own conclusions and those of others on the probable significance of findings published before 1940. "The main histological changes in toxemia are found in the placenta, liver, kidneys and retina. The placental changes consist of a premature degeneration of the syncytium. These lesions develop early and may precede even the appearance of albuminuria. The present evidence points to the placenta as the 'intrauterine factor' responsible for toxemia of pregnancy. Although the mechanism by which toxemia is produced is not known in its details, a chemical (hormonal) mechanism . . . may be suspected with some justification. The clinical predisposing factors . . . disturb the placental circulation, causing secondary degenerative changes (premature ageing). The observations reported by us and others regarding the origin of the edema of pregnancy indicate that . . . the abnormalities in water metabolism during pregnancy are not due to causes usually found in the non-pregnant state. A consideration of the various possible explanations . . . suggests specific factors which retain fluid in cells and tissue spaces. By exclusion, endocrine factors such as exist in premenstrual edema and Cushing's syndrome can be suspected. It is possible that the placenta produces a water-retaining hormone and also a vasoconstrictor substance. . . . "

PLACENTAL HORMONES. *Chorionic gonadotropin.* A gonadotropic factor in the urine of pregnant women was discovered by Ascheim and Zondek (7). That it differs from gonadotropic hormones of anterior pituitary origin was demonstrated by Evans *et al.* (56, 57). By tissue culture technic, Gey *et al.* (62, 81) showed it to be a placental product, probably from the cytotrophoblast. The histochemical studies of Wislocki and Bennett (206) also indicated that the cytotrophoblast of the chorionic villi is responsible for its secretion. These latter investigators also pointed out that the growth and activity of the cytotrophoblast are roughly in accord with the previosuly established curves of gonadotropic content in blood and urine throughout gestation (27, 55, 134, 142, 161, 209). Chorionic gonadotropin, or CG, is now the generally accepted appellation for the hormone.

Ehrhardt (52) reported positive tests for gonadotropic hormone in the cerebrospinal fluid of eclamptic and pre-eclamptic women, but normal titers in their blood and urine, and concluded therefrom that the gonadotropic activity of the anterior pituitary was high in toxemia of late pregnancy. Excessive gonadotropic hormone in the blood and urine of patients with toxemia was first reported by Smith and Smith (137–139), who demonstrated also that the placentas of of toxemic patients contained more than could be recovered from those of normal pregnancy and that the high gonadotropic titer of blood and placenta in toxemia was not accountable to pituitary gonadotropes (140). High CG in the blood and urine of women with toxemia has been confirmed (5, 21, 70, 119, 134, 185), as well as the evidence for its placental origin (5). It has been shown, however, not to be demonstrable in all cases, not to bear any direct relation to the severity of the disease and to be associated also with stillbirth and premature delivery in the absence of toxic signs (120, 145, 148, 171, 193). Browne, Henry and Venning (29) could make no correlation between elevated CG and late pregnancy toxemia. Taylor and Scadron (180) and Cohen, Wilson and Brenan (34) found only a slight correlation. The latter investigators studied 119 pregnancies and based their conclusion on the fact that, although 44 per cent of toxemic patients had high serum CG, elevated levels were also present in 15 per cent of normal deliveries. Other studies (120, 148, 161, 171) have indicated that elevation of CG during the last month before labor is a normal phenomenon. This may explain the high values in normal late pregnancy which disturbed Taylor and Scadron and Cohen *et al.*

Smith and Smith, in 1934 (138), noted a marked rise of serum CG early in the third trimester in a woman who later developed pre-eclampsia. They have since performed repeated analyses for serum CG upon 117 women during the last trimester to determine whether or not accidents of late pregnancy could be predicted by an abnormal rise in this factor (139, 144–146, 148, 161, 171). (Since the incidence of late pregnancy accidents was known to be high in patients with diabetes, 68 of the above number were chosen for study because they had diabetes.) In all of 42 women whose pregnancies progressed and terminated normally, serum CG was at a low and uniform level between the twenty-fourth and thirty-sixth weeks, any rise after the thirty-sixth week being considered normal. Of 56 patients in whom a progressive elevation of CG was observed

prior to the thirty-sixth week, 45 developed toxemia, eight delivered prematurely and three had their babies die *in utero*. On the other hand, 19 patients in whom no abnormal elevation of serum CG was detected developed late pregnancy complications. Nine had stillbirth or premature delivery and 10 developed toxemia. They concluded that, although an abnormal rise of this hormone in the blood prior to the last month of pregnancy warrants the prediction of later trouble, it gives no indication of the type or severity of the accident and, furthermore, that accidents may occur without any warning so far as a rise of CG is concerned.

High serum CG prior to the appearance of toxic signs was reported by Rakoff (119) in two of three patients. White *et al.* (200–203) performed repeated analyses for serum CG as a prognostic test in patients with diabetes complicated by pregnancy. In 1945 she (200) summarized results concerning 181 such women. The studies on the first 33 of her patients were performed by Smith and Smith (144, 145, 161); therefore these cases were included with the 117 just analysed (*v.s.*). Her results agreed with the Smiths' in that an abnormal rise of serum CG was always followed by clinical abnormalities and the level of hormone was consistently low in uncomplicated pregnancies. She failed to confirm, however, the fairly high incidence of late pregnancy accidents observed by the Smiths in patients whose serum CG did not become abnormally elevated. Only one of her 52 patients with normal CG levels developed toxemia, none delivered prematurely and only 2 lost their babies. Rubin, Dorfman and Miller (120) followed serum CG during the last trimester in 5 diabetic patients, one of whom had normal CG levels and an uncomplicated pregnancy, 2 of whom developed toxemia, 1 with and 1 without high CG, and 2 of whom had fetal death, 1 with and 1 without elevated CG.

Progesterone. Progestational activity has been repeatedly detected in extracts of human placenta (1, 53, 54, 65, 101, 152). Since histochemical studies (42, 206) indicate that the chorionic syncytium elaborates steroids, progesterone is probably secreted by these cells. The demonstration that pregnanediol glucuronidate is an excretory product of progesterone (190) and the development of a method of quantification (189) led to extensive studies concerning the urinary excretion of this compound in normal and toxemic pregnancy. In normal pregnancy the amount increases from around 10.0 mgm. daily at one month to between 60.0 and 130.0 mgm. daily shortly before term and practically disappears within 24 hours of delivery (10, 12, 28–30, 32, 66, 80, 144, 146–148). The corpus luteum has been removed in pregnancy without more than slight and temporary alteration in the excretion of pregnanediol (28, 79). Browne and his associates (30) noted peaking of pregnanediol excretion at approximately four-week intervals during pregnancy. This was also observed by Smith and Smith (146), who found in individual curves the final and highest peak about two weeks before term, this being followed by decreasing values before and during labor (147). A prepartum drop in pregnanediol was also noted by others (10, 12, 13, 66). Studies by Stover and Pratt on 5 patients (175), by Wilson, Randall and Osterberg on 1 patient (205) and by Bachman on 6 women (9)

failed to reveal any decline of pregnanediol before labor, but Lyon (94), reporting results on 68 patients whose labors were spontaneous, determined a peak of pregnanediol excretion about two weeks before delivery, followed by decreasing values and then a precipitous drop for five to seven days prepartum, regardless of whether labor was premature, at term or postmature.

Smith and Smith (135, 136), in their earliest studies of estrogen and progesterone metabolism, discovered that progesterone decreased the destruction of both administered and secreted estrogen. Their finding of low estrogens in toxemia (*v.i.*) led them to suspect, therefore, that progesterone might also be deficient (139, 161). Smith and Kennard (152) attempted to compare the progesterone content of normal and toxemic placentas. Although there was evidence of deficient progesterone in half of the placentas from untreated toxemic patients, the method used was not sufficiently accurate to warrant definite conclusions. In 1938 three groups of investigators, working independently, reported low values for urinary pregnanediol in toxemia of late pregnancy (29, 144, 194). This has been confirmed (11, 13, 146, 148, 180). There is general agreement, however, that the abnormality is revealed only by comparing the averages of normal with those of toxemic pregnancy. Cope (39, 40) did not find it in 10 women with late pregnancy toxemia and Hain (66) observed excessively high urinary pregnanediol in 1 patient with severe pre-eclampsia.

Smith and Smith (148) pointed out the wide range of normal values and concluded that the curve of pregnanediol excretion as pregnancy advances is more inportant than the actual level at any given time. In 14 normal pregnancies they found progressively increasing values, with minor fluctuations, between the twenty-eighth and thirty-sixth weeks; whereas, in 21 patients with toxemia the values steadily decreased during this time, with one exception, in a patient whose toxemia became self-corrected during the period of observation. In two women studied prior to the onset of toxic signs, urinary pregnanediol progressively diminished for four weeks before any clinical abnormality was apparent. These investigators have studied the relationship between progesterone and the urinary excretion of estrogen metabolites (*v.i.* Metabolism of the estrogens) and believe that measurement of the latter, though more laborious, provides more information concerning secreted progesterone than does the determination of urinary pregnanediol. In all of 50 patients studied by them prior to the development of toxemia, premature delivery or intrauterine death, evidence for a progressive deficiency of progesterone before as well as during the clinical abnormality was acquired (144, 146, 148, 149, 171, 172). White and Hunt (202) utilized pregnanediol determinations as a prognostic test in 60 diabetic women of whom 43 were receiving therapy with estrogen and progesterone. Of the untreated patients, 12 had normal curves of pregnanediol excretion and normal pregnancies with live births, though two of the babies died after delivery. Of the five untreated women whose pregnanediol excretion was decreasing between the twenty-fifth and thirty-sixth weeks, three developed toxemia, one delivered prematurely and the fifth showed no clinical abnormality before being delivered by cesarean section in the thirty-fourth week. Rubin, Dorfman and Miller (120)

noted diminished excretion of pregnanediol prior to toxemia in two of their diabetic patients but failed to find this abnormality in two others whose babies died, one in utero and the other after delivery.

The estrogens. Estrogenic substance was first demonstrated in the human placenta by Fellner (60). This has been adequately confirmed (3). In 1927, Ascheim and Zondek (7) discovered the great concentration of estrogenic material in the urine of pregnant women, and Margaret Smith (157) found increasing amounts of it in the blood throughout human pregnancy. Removal of the corpus luteum or of all ovarian tissue as early as the sixth to eighth week of pregnancy has been shown not to interrupt gestation (2, 4, 8, 22, 28, 49, 50, 79, 118, 123, 167, 177, 191) or the continued elaboration of estrogen (2, 4, 22, 118, 123, 167, 191). The concentration of estrogens in the placenta, their continued excretion after ovariectomy,and the prompt cessation of excretion after delivery contribute to the generally accepted tenet that the placenta forms estrogens; the site of formation being likely in the syncytial cells of the chorionic villi (42, 206).

Estrogenic activity in the serum is relatively low until after the second month of normal pregnancy. It increases rapidly thereafter, the highest values being acquired during the last month (138, 139, 157, 161). Quantification of blood estrogen is not satisfactory because of still unsolved technical difficulties. The discovery that all but a small part of urinary estrogen is in combined forms, relatively inactive biologically and insoluble in water-immiscible organic solvents (20, 35, 90, 141, 210) made earlier studies of estrogen excretion only roughly significant. Subsequent investigations, in which hydrolysis and improved methods of extraction were used, yielded more accurate and significant results and established that the total estrogenic potency of the urine rises before the first missed period (100, 142), increases gradually and then markedly during pregnancy, reaching a peak in the last month, and diminishes rapidly prior to the onset of labor, becoming negligible within three days after delivery (9, 27, 29, 36, 46, 66, 93, 98, 134, 144, 146–148, 154, 161).

Low levels of estrogen in the blood, urine and placentas of most patients with toxemia of late pregnancy were first reported by Smith and Smith (137–140, 161). This finding, as regards the urine, has been confirmed by nearly all investigators (29, 93, 119, 120, 124, 134, 144, 146, 148, 149, 170, 171, 180, 193). There is, however, general agreement that in individual cases values within or even above the normal range often are obtained. Heim (70) reported high and Hain (66), in one case, very high urinary estrogen in toxemia, and normal blood values have been described (16). Shute (129–133), using antiproteolytic power of serum as a gauge of its estrogen content, claimed both high and low estrogen in toxemia. As with urinary pregnanediol, the curve of excretion in each individual is thought by the Smiths to be more important than the actual level at any one time. This is apparent in their curves for total estrogen of blood and urine in diabetic and non-diabetic women (161). Seven who developed toxemia or had premature delivery failed to show the marked increase of estrogen in blood and urine between the sixth and eighth months that characterized the 17

normal curves. In their later studies, in which urinary estrogen was separated into estradiol, estrone and estriol and the values converted from rat units into milligrams, a progressive decrease of total estrogen was demonstrated before and during toxemia, premature delivery and intrauterine death in all of 50 patients studied (144, 146, 148, 149, 171, 172).

That decreasing excretion of estrogens and pregnanediol before labor and late pregnancy toxemia actually reflects decreasing secretion of placental steroids is indicated by histological and histochemical studies of human placentas. The syncytial degeneration which characterizes normal term placentas involves an almost complete disappearance of the lipoidal droplets associated with secretion of steroid hormones (42, 206, 207). In 1936, Tenney (184) discovered that syncytial degeneration similar to but more pronounced than that of term placentas is of consistent occurrence in toxemic placentas. He and Parker later (186), from an examination of 100 toxemic placentas, correlated the amount of syncytial degeneration with the severity of the disease and concluded that "placenta damage begins before clinical signs of the condition appear." Wislocki and Dempsey (207) confirmed these observations in two toxemic placentas delivered in the fifth month, finding histochemical, syncytial changes typical of the organ at term. (They also noted a decrease of cytoplasmic basophilia and a premature increase of phosphatases suggesting a possible disturbance of nucleoprotein metabolism in this disease.) Histological and histochemical evidence, therefore, substantiates the above hormonal evidence, pointing to a premature aging of the placenta in toxemia and therefrom a premature deficiency in the secretion of progesterone and estrogen. An actual deficiency, then, of estrogen and progesterone, rather than any renal retention or change in conjugation to account for the low urinary levels, appears to be well established. The theory introduced by a number of workers (12, 29, 66, 179, 180, 182) that toxic signs, particularly changes in electrolyte balance and water retention, are due to high levels of these steroid hormones must, therefore, be discarded.

Smith and Smith (145) in summarizing and evaluating their findings on CG in the blood and urine during normal and toxemic pregnancy, postulated utilization of this hormone in the placenta for the production of estrogen and progesterone. They had noted that the precipitous decrease in CG of blood and urine at around the twelfth week of normal pregnancy was accompanied by a marked increase in estrogen excretion (142) and that the prepartum decrease in estrogen excretion coincided with a rise in serum CG (154, 161). This same reciprocal relationship between CG and estrogens had been observed in late pregnancy toxemia (144, 161), suggesting to them that an abnormal elevation of serum CG in this condition and at term reflected failing utilization. To explain the absence of elevated serum CG in some patients with toxemia, they postulated an actual decrease in the secretion of this factor from placental damage. In support of these hypotheses is the following more recent evidence. The cytotrophoblast and its more differentiated derivatives, the cells of Langhans, are the likely source of CG (81, 206). It is well known that the Langhans cells become fewer in number as pregnancy advances and are practically absent in

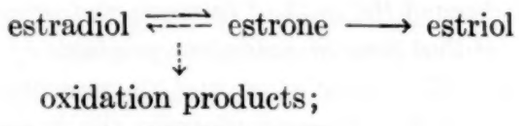
term placentas. There is no increase of cytotrophoblast in toxemic placentas (72, 207). And, finally, in two such placentas delivered in the second trimester Wislocki and Dempsey (207) found premature regression of the Langhans cells as well as the syncytium. As pregnancy continues, therefore, whether normal or abnormal, there is no proliferation of the cells which probably secrete CG to account for high values at term and in toxemia but rather, if anything, a degeneration. In view of the histochemical and hormonal findings pointing to decreased sex steroid production at term and in toxemia, then, it seems possible that failing utilization of CG accounts for the larger amounts of it in the circulation at these times and that decreased elaboration as well as failing utilization account for the normal serum values sometimes acquired. This concept was further corroborated recently (168) by a comparison of hormonal values and placental pathology in the same patients.

Metabolism of the estrogens. Certain changes in the partition of urinary estrogens and in the excretion of what appear to be estrogen metabolites have been found to precede and accompany the onset of labor and of late pregnancy toxemia. In order to evaluate the significance of these, a brief review on the subject of estrogen metabolism is in order.

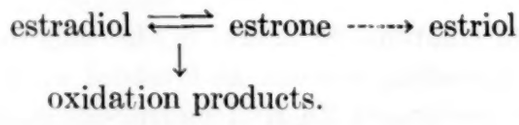
Three estrogens have been identified in the urine of pregnant women: estrone (31, 48), estriol (47, 96) and α estradiol (77, 153). Of these, α estradiol is commonly regarded as the primary estrogen in humans from which the others are derived. Marrian (97) suggested that estriol may also be secreted by the human placenta. In 1937 Pincus and Zahl (116) injected the three estrogens separately into rabbits under varying conditions and determined their subsequent urinary excretion by separation and colorimetric assay. They concluded, as had been previously demonstrated both in rabbits and women (135, 136) that progesterone increases the amount of estrogen excreted, probably by inhibiting destruction. Moreover, their results indicated the following scheme of conversion within the animal body: α estradiol $\leftrightharpoons$ estrone $\rightarrow$ estriol, the estrone to estriol reaction being facilitated by the presence of progesterone. That the same metabolic relationships pertain in women seems likely from the studies of Smith and Smith and their associates (144, 146–149, 155, 156, 170, 173) and those of Pincus and his associates (113–115). These conclusions are based entirely upon colorimetric (Pincus) and biological (Smith and Smith) assay of the three estrogens separated by admittedly imperfect methods. Chemical confirmation of them in man, however, was at least partially acquired by the isolation of α estradiol and estriol in the urine following estrone administration (110, 111) and of estrone after the administration of α estradiol (68, 69).

All investigators have been impressed by the small percentage of injected estrogen recoverable in the urine under any conditions. Numerous attempts have been made to isolate compounds in human urine which could be identified as products of the endogenous inactivation of the estrogens but none of them has been successful. Smith and Smith (143) evolved a method of Zn-HCl hydrolysis of human urine which results in much more estrogenic activity than can be accounted for by the known estrogens present. The additional activity appears

to be derived from reduction into active forms of estrogenically inactive oxidation products of the estrogens. Since the precursors of this additional potency have not yet been identified, this deduction is based largely upon circumstantial evidence. The procedure, however, seems to provide a gauge of the rate of oxidation of estrogens within the body (155, 156, 162, 173). Using this method in conjunction with the separate assay of estradiol, estrone and estriol fractions, they strengthened the evidence that progesterone decreases the rate of estrogen inactivation, by facilitating the conversion of estrone, which is rapidly inactivated *in vivo*, to estriol, which is much less readily destroyed, according to recovery experiments (126, 156). Their results also indicated that the destructive mechanism inhibited by progesterone is an oxidative one related largely to the reversible estradiol to estrone reaction. Thus in the presence of adequate progesterone:



whereas, when progesterone is deficient,



In applying these methods to the study of estrogen and progesterone metabolism in both pregnant and non-pregnant women, they were led to the concept that estrogenically inactive oxidation products of the reversible estradiol to estrone reaction, rather than estrogens *per se*, stimulate the production of estrogen and progesterone, this being accomplished in the non-pregnant woman through release of pituitary gonadotropes and in pregnancy through increased utilization of CG (147, 148, 150, 156, 166, 171, 173). This concept was strengthened by the experimental demonstration, 1, that a lactone produced from crystalline estrone by oxidative inactivation (198) causes release of pituitary gonadotropes; whereas, estrone has this property only under conditions involving rapid inactivation *in vivo* (158-160); 2, that diethylstilbestrol behaves like the estrone-lactone (163) and, 3, causes increased excretion of pregnanediol together with a drop in serum CG in pregnant women (168, 172).

In 38 untreated pregnancies the Smiths performed repeated urinalyses for estradiol, estrone, estriol and the additional estrogenic potency recoverable after Zn-HCl hydrolysis (148, 150, 171). Biological assay was used and the Zn-HCl activity interpreted as a gauge of the rate of oxidative inactivation of the estrogens *in vivo*. All of these women were studied from early in the third trimester to delivery. Of the 23 (see second paragraph below) who developed pre-eclampsia or eclampsia or had intrauterine death or premature delivery during the period of observation, three had been followed during the second trimester also. In 6 of the 15 normal pregnancies analyses were performed from the twelfth week to term. In normal pregnancy the percentage of total estrogens accountable to estriol was found to increase steadily, with minor

fluctuations, reaching a peak at approximately two weeks before term. At this time estrone was also high, whereas the proportion of activity accountable to estradiol was low, and the Zn-HCl values indicated the lowest rate of oxidative inactivation found at any time of pregnancy. These findings, together with the thirty-eighth-week peak in pregnanediol excretion were interpreted as reflecting a maximum rate of conversion of estradiol to estrone to estriol and a minimum rate of oxidative inactivation of the estrogens due to a high and balanced rate of secretion of estrogens and progesterone. The subsequent prepartum drop in estriol and estrone accompanied by an increase in estradiol and in the rate of estrogen oxidation was interpreted as reflecting decreased conversion and increased destruction of the estrogens due to progesterone withdrawal as well as reduced secretion of estrogen. According to their concept, *the syncytial degeneration of the placenta during these last weeks with decreasing secretion of steroids has its incipience at the time of the peak of estrogen and progesterone production and is due to the deficiency at that time of oxidation products of the estrogens and hence a deficient utilization of CG.* Once syncytial degeneration is under way, they believe, the process cannot be reversed, despite the increasing rate of estrogen inactivation (150, 168).

Bachman (9), using colorimetric assay, is the only other investigator who separately measured estradiol, estrone and estriol in the urines of pregnant women. No one has performed Zn-HCl hydrolysis in conjunction with such studies, and Bachman's investigations were limited to six normal pregnancies followed during the last trimester. His findings agree with the Smiths' as regards estradiol and estriol, but he failed to observe any prepartum drop in estrone.

Changes in estradiol, estrone and estriol and in the rate of estrogen inactivation similar to those during the last two weeks of normal pregnancy were consistently found by the Smiths to precede and accompany late pregnancy toxemia and associated accidents in the 23 abnormal cases studied during the last trimester the only difference being that when they take place prematurely they progress over a longer period and become exaggerated to a degree normally attained only after labor is well advanced. In the three patients followed from earlier in pregnancy there was urinary evidence of a premature peak in estrogen and progesterone secretion around the twenty-eighth week and a comparative *deficiency of estrogen oxidation products throughout the second trimester.* These patients were diabetic and, as pointed out, the earlier hormonal abnormality may not precede the premature degenerative changes in the placental syncytium in all cases of late pregnancy toxemia. Such a deficiency of oxidation products during the second trimester, however, may be the predisposing factor in a certain percentage of patients just as, according to their concept, a similar situation during the last weeks of normal pregnancy is responsible for failing utilization of CG and the prepartum withdrawal of placental steroids.

THEORIES OF ETIOLOGY AND ATTEMPTS AT TREATMENT. *The thyroid gland.* On the theory that hypothyroidism accompanied by hypercholesteremia produces placental arterial disease predisposing to thrombosis, infarcts and degene-

ation with resultant absorption of toxins from degenerated placental tissue, Patterson *et al.* (109) administered thyroid as a preventive measure. The clinical results reported by them and others (38, 64, 78, 86) with thyroid or iodine have not indicated specificity of therapy. As stated above, Dexter and Weiss (43) concluded that hypofunction of this gland cannot play a significant rôle in the causation of late pregnancy toxemia.

The adrenal glands. Parks (106, 107), assuming that the upset of sodium and potassium in toxemia is due to excessive hormones from the fetal adrenal glands, reported the control of edema by potassium in patients who had not responded to a low intake of sodium chloride.

Progesterone. Robson and Paterson (108, 122), from experiments with rabbits, concluded that the toxic condition from the uterus might be due to a failure of placental nutrition following removal of luteal secretion and described encouraging clinical results from small amounts of progesterone given early in the disease. These were not consistently achieved by others (14, 99, 103).

Estrogens. Shute (129–133), on the basis of a blood estrogen test devised by himself, classified toxemic patients into two groups, those with low estrogen (true pre-eclampsia and eclampsia) and those with high estrogen. The former he treated with estrogen and the latter with vitamin E, with satisfactory control of both. There are not reports in the literature of attempts to confirm the specificity of his test or his therapeutic results.

Testing their hypothesis that oxidation products of the estrogens cause utilization of CG for the placental secretion of its steroids, Smith and Smith (168, 172) investigated the effect of preventive treatment with diethylstilbestrol, this drug being employed because oxidation products such as estrone-lactone (*v.s.*) were not available in sufficient amounts and because experiments (163) had indicated a superiority of diethylstilbestrol for this purpose. Two diabetic patients with bad obstetrical histories were studied both during treatment and control periods and the placenta of one examined histologically. From the results, utilization of CG was enhanced and a premature aging of the placenta in steroid secretion, as well as late pregnancy complications, were averted. They (168) demonstrated that, once toxemia is evident, therapy with diethylstilbestrol is not likely to stimulate secretion of placental steroids or have any clearly favorable effect on the disease, since an excess of endogenous oxidation products pertains by this time and is unable itself to bring on renewed secretory activity in the already degenerate syncytium.[1]

[1] Since this paper went to press, Davis and Fugo (DAVIS, M. E., AND N. W. FUGO. Proc. Soc. Exper. Biol. and Med. **65**: 283, 1947) have reported experiments in which 50 to 200 mgm. of diethylstilbestrol were administered daily by mouth starting at the fourth to tenth week of pregnancy and continued for 6 to 8 weeks. This dosage is greatly in excess of the amounts recommended by Smith and Smith for early pregnancy (168, 172). In normal untreated pregnancy pregnanediol excretion increases steadily as pregnancy progresses; whereas the results presented by Davis and Fugo show no increase during the period of stilbestrol administration. It seems possible that such large doses early in pregnancy might inhibit progesterone secretion through depressing the utilization of CG, just as the prolonged administration of large doses to the non-pregnant woman are known to inhibit ovarian secretion through depressing pituitary gonadotropic activity.

Progesterone and estrogen. On the basis of their evidence for a progressive deficiency of these two hormones before and during late pregnancy toxemia and associated accidents, the clinical and hormonological effect of replacement therapy was investigated by Smith and Smith (144, 146, 149, 170). As a preventive measure in patients in whom studies of blood and urine warranted the prediction of later trouble, the therapy was considered promising but impractical. The first 10 trials of preventive therapy with estrogen and progesterone in diabetic patients at the George F. Baker Clinic of the New England Deaconess Hospital were carried out by the Smiths and reported by them (144, 146), as well as by White and her associates (200–203). The latter continued with this form of therapy and described good results in 91 patients, particularly as regards reduction of fetal mortality (200). There are no other publications concerning the use of such therapy prophylactically in either diabetic or non-diabetic patients, probably because of the expense involved. The prophylactic stimulation of placental secretion of sex steroids by diethylstilbestrol administration, starting prior to any evidence of hormonal deficiency, now being investigated by Smith and Smith, would appear to offer a more physiological and practical approach.

Definitive therapy of toxemia with estrogen and progesterone has been disappointing even when seemingly large amounts were given (144, 146, 149, 170 179). Taylor (179) considered this to be evidence against steroid deficiency as solely responsible for the disease. Smith and Smith (149) demonstrated some temporary alleviation of pre-eclampsia with estrogen and progesterone, but their hormonal findings indicated that once toxic signs appear there is a rapid increase in the rate of destruction of administered as well as secreted hormones. This phenomenon, as well as decreased secretion, they believe, contributes to the low levels in toxemia. With Taylor they consider the deficiency of estrogen and progesterone not to be the final precipitating cause of the toxemic syndrome. Its universal occurrence in all cases, however, as shown by hormonal and histological studies (*v.s.*) and the evidence that it precedes toxic signs implicate it as an intermediary and contributory factor.

Menstruation and toxemia. In the publications covered in this review, the Smiths repeatedly referred to similarities between the phenomenon of menstruation and toxemia of late pregnancy, the analogy having been suggested to them primarily because of their evidence from urinalysis for a hormonal situation before and during menstruation entirely like that before and during late pregnancy accidents. The similarity between premenstrual changes and toxemia were observed clinically by Dexter and Weiss (43), who stated further that so far as types of edema and symptoms are concerned there is a certain similarity in menstruation, normal pregnancy and toxemia. The Smiths (150) recently summarized their evidence that withdrawal of hormonal support from the pregnant as from the non-pregnant uterus results in the formation and release of a toxic metabolite of tissue catabolism like the toxin they found in the menstruating endometrium. The pathological effects of this toxin upon experimental animals are such as to warrant the assumption that it is directly responsible for the local

changes resulting in menstruation and for the similar but generalized damage of late pregnancy toxemia. This hypothesis is strengthened by their evidence for certain properties in the circulating blood of women with toxemia similar to those of menstrual discharge, *viz.*, pyrogenic and fibrinolytic activity,[2] precipitation by anti-canine necrosin rabbit serum and a pseudoglobulin fraction capable of prolonging the survival time of rats given a lethal dose of the toxic euglobulin fraction of menstrual discharge (151, 165, 169), these properties being absent in normal pregnancy except during prolonged labor. According to their theory, the maternal portion of the placenta, *i.e.*, the decidua, being endometrium, should be the source of the toxin. (As such it could also be the cause of postpartum eclampsia.) A limited number of tests with specimens of decidua removed at the time of cesarean section have revealed marked toxicity in those from pre-eclamptic and eclamptic patients (150). In their publications, they have emphasized the extreme lability of the menstrual toxin (169). Failure to consider this possibility may explain the failures of the past to demonstrate any specific or consistent toxicity in placentas from toxemic women. Hertig (72) finds decidual necrosis and vascular thrombosis in relation to delivery and toxemia.

Certain known interrelationships between vascular supply, hormone production and tissue metabolism suggest that various conditions would bring about a menstrual-like phenomenon in the pregnant uterus. In the section on the metabolism of the estrogens (*v.s.*) the Smiths' data, suggesting that a deficiency of estrogen oxidation products during the second trimester might lead to premature syncytial degeneration was reviewed. Decidual, like endometrial, catabolism would be expected to result from the consequent withdrawal of estrogen and progesterone, with the production of toxin. The toxin in turn, to judge from its action in experimental animals, would be expected to augment degeneration of the syncytium and even cause degeneration of the Langhans cells and cytotrophoblast, thus disturbing hormone production even further.

Page (105-A) pointed out the possible rôle of placental ischemia in the pathogenesis of toxemia. An adequate vascular supply to the placenta is contingent upon an adequate production of estrogen and progesterone. Conversely, the Smiths (147) showed that decreased blood supply to the placenta through uterine contractions depresses the secretion of the steroid hormones. Decreased blood-supply to the uterine contents, such as might result from local mechanical conditions or generalized vascular situations, would be expected to result in degeneration not only of the syncytium but also of the decidua. Furthermore, since the pathological effect of menstrual toxin reflects vasoconstriction (151, 169), its release would in itself interfere with vascular supply.

In the course of their studies, the Smiths (151, 164, 169) deduced that types of injury not related to withdrawal of hormonal support cause the release of a similar if not identical toxin and acquired evidence for its presence in human exudative material and in the circulating blood of women with damaged tissues.

[2] Fibrinolytic activity in the circulating blood of patients with late pregnancy toxemia has been confirmed (203-A).

On this basis, damage of the chorionic epithelium might contribute to the production of toxin.

Any one of a number of processes, therefore, might be primarily responsible for setting off a chain of events leading to an overwhelming production of toxin and to toxemia of late pregnancy, unless delivery of the uterine contents intervened. Whatever the primary etiology, the final syndrome would be the same, a vicious circle in which decrease of vascular supply, hormonal deficiency and toxin formation were augmenting one another.

Even in cases wherein a deficiency of oxidation products of the estrogens does not pertain during the second trimester, the prophylactic use of adequate dosages of stilbestrol, now being investigated by Smith and Smith may prove effective through increasing uterine vascularity, both *per se* and by stimulating steroid secretion in the placenta. Once the disease is clinically manifest, according to their findings, the vicious circle of the final syndrome is already established and the best hope of cutting in upon it, aside from delivery of the products of conception, lies in neutralization of the toxin. To investigate this possibility, they (168) studied a few cases of pre-eclampsia to whom a crude preparation of their protective pseudoglobulin was experimentally administered. Clinically the results could be considered no more than promising. Their principal interest lies in the theoretical implications which appear to uphold the above concepts.

The presence of a toxin in toxemia has always offered attractive possibilities for explaining the clinical syndrome. Complete studies on the effects of menstrual toxin remain to be performed but experiments reported show that it causes edema and vasoconstriction and indicate that it produces damage of capillary endothelium as well as of tissues in general and has an antidiuretic effect (169). Dexter and Weiss (9) offered evidence for the existence of generalized damage of the capillary endothelial system in toxemia. That the toxin may be responsible for the hypertension and proteinuria of toxemia remains to be investigated. Damage of the renal glomerular capillaries would explain the proteinuria. The hypertension may be the consequence of a number of effects of the toxin, renal, suprarenal, cerebral and direct upon blood vessels. The antidiuretic substance in the placentas of toxemic patients (67) may be the toxin working directly or through pituitary stimulation (*v.i.*) in the experimental animal. The same explanation may apply as regards the melanophore-expanding principle in the blood and placentas of patients with toxemia (87). That patients with toxemia are hypersensitive to injected posterior-lobe hormones (*v.s.* Effects of Administered Posterior Pituitary Extracts) suggests an effect superimposed on that of the toxin. Smith and Smith (169) showed in rats that menstrual toxin causes the release of pituitary gonadotropic and adrenotropic hormones and believe that it produces an "alarm" reaction involving quite possibly the release of other pituitary hormones. Epinephrinemia in eclampsia (95) may reflect such an "alarm" reaction, and pituitary stimulation may explain a number of observations made in preeclamptic and eclamptic patients, *viz.*, urinary thyrotropic hormone (19), possibly the urinary antidiuretic substance (*v.s.* Antidiuretic substance in the urine), thyroid hormone (75) and, particularly, the possibility considered by some of over-

activity of the adrenal cortex (26, 43, 181). None of these abnormalities has been demonstrated prior to the clinical onset of the disease.

SUMMARY

From the above it is apparent that the primary etiology of toxemia of late pregnancy has yet to be determined, that no definitive treatment has been rewarded with spectacular cure, such as usually occurs following delivery of the products of conception, and that prevention of the disease by the administration of hormones has yet to be proven. Certain aberrations from the normal in hormonal or hormonal-like findings and in placental morphology appear to be established in women with pre-eclampsia and eclampsia: 1, excessive antidiuretic substance in urine and placenta; 2, a melanophore-expanding principle in the blood and placenta; 3, a hypersensitivity to the injection of posterior pituitary secretions; 4, high titers of chorionic gonadotropin in a large percentage of cases; 5, decreased excretion of estrogens and pregnanediol, and 6, histological and histochemical changes in the placenta similar to but more marked than those of the normal placenta at term and primarily involving degeneration of the syncytial cells which probably secrete the steroid hormones. Of these established abnormalities, the last three are underway prior to clinical manifestations and occur normally at term.

Synthesis. That the primary etiology of toxemia may consist of a number of causes is suggested by the already familiar predisposing factors such as essential hypertension, diabetes, primigravidity, hydatidiform mole, twins and hydramnios. The evidence at hand appears to establish premature senility of the placental syncytium and premature withdrawal of the placental steroid hormones as the final intermediary pathology. This disturbance, which occurs normally at term, must be brought about prematurely by the working of the primary etiology, which probably involves either an intrinsic metabolic abnormality affecting the placenta or a decrease in blood supply to the placenta or both. Since the syncytial-steroid aberration from the normal characterizes all cases, it may logically be assumed to be contributory factor to the development of toxemia in all cases. From the above discussion (*v.s. Theories of etiology and attempts at treatment*), however, it cannot be assigned the rôle of the sole precipitating cause of the toxemic syndrome. Recent incomplete work suggests, 1, that withdrawal of hormonal support from the pregnant as from the non-pregnant uterus may result in the formation of a menstrual-like toxin in the placenta (? maternal portion, *i.e.*, decidua); 2, that the primary etiology may, in conjunction with the steroid deprivation it causes, do the same thing, and 3, that release of this toxin may prove to be the final cause of toxemia of late pregnancy.

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LOCAL ACTION OF SEX HORMONES

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THE HYPODERMIC SYRINGE was, until recently, the standard medium for the administration of sex hormones to experimental animals and to human patients. The discoveries that some sex hormones are active orally and that sexual stimulation can be effected by hormonal pellets implanted under the skin (Deanesly and Parkes, 1937) or placed under the tongue are products of only the past few years. It is also possible to elicit the characteristic tissue response to a hormone by bringing it into direct contact with the target organ. Many demonstrations have been made of the effectiveness of sex hormones when applied locally in this manner. This paper is a review of the published reports of laboratory and clinical experiences with local organotherapy with the so-called male and female hormones.

VAGINA. *Experimental.* The intravaginal instillation of estrogens and biological materials containing estrogens has shown the feasibility of inducing vaginal estrus by this means. When the instilled quantities of hormone are sufficiently small their action is strictly a local one. Robson and Adler (1940) demonstrated this fact by means of a cleverly planned experiment in which they separated the upper part of the mouse vagina from the lower part. The latter is known to be more responsive to estrogens; but when estradiol, estriol glucuronide, or stilbestrol were injected into the upper vaginal sac, a distinctly greater estrogenic effect was produced there. It was concluded, therefore, that the action was local rather than systemic. In castrated female rats vaginally administered stilbestrol, in amounts up to 1 gamma daily, produced vaginal cornification but was without effect in modifying the appearance of castration cells in the pituitary (Morrell and Hart, 1941). Estrous changes in strips of castrated rat vagina *in vitro* were reported by Berger (1937) following the addition of estrogen to the culture medium. These experiments conclusively eliminated the possibility of systemic influences.

Interest in the local vaginal action of estrogens in experimental animals is based largely upon its usefulness for assay techniques. Castrated adult rats, and to a lesser extent mice, have been used for this purpose. Experiments have shown that bioassay methods for estrogens based on the intravaginal route of administration are simple, reliable and in general more sensitive than other biological methods of assay. The intravaginal method offers the further

advantage that it permits the analysis of estrogens in a variety of vehicles including pessaries (Powers, Varley and Morrell, 1929), cotton pledgets (Palmer and Zuckerman, 1939), blood (Albrieux, 1941), oil and other solvents. It also results in an economy of animals, since the same individuals can be used over and over again.

Using cotton wool pledgets soaked with stilbestrol in oil, Palmer and Zuckerman (1939) observed estrous changes in the vagina of mice within 24 hours after the administration of as little as 0.05 gamma of stilbestrol. Lyons and Templeton (1936) and Yerby (1937) assayed estrogens by instilling urinary extracts vaginally in castrated adult rats and observing the degree of cornification 24 hours after the second of 2 daily doses. Blood estrogens were assayed intravaginally in rats by Albrieux (1941), using pellets made from desiccated blood. Markee and Berg (1942), employing mice for the assay of blood estrogens during the menstrual cycle, found that extracts containing as little as 0.015 I.U. of estrone, when instilled vaginally, induced macroscopically detectable vaginal mucus.

Estimates of the relative effectiveness on the vaginal mucosa of estrogens applied locally as compared with estrogens administered by subcutaneous injection vary widely. Differences resulting from these two methods of administration depend upon the species and age of the test animal as well as the specific estrogenic compound and its vehicle, and are accentuated by variations in the dosage, frequency of treatment and time of observation. Powers, Varley and Morrell (1929) reported a ratio of effective subcutaneous dose to effective vaginal dose of 1:3, Pratt and Smeltzer (1929) 1:2, in spayed rats treated with estrogen. Subsequent investigators, however, have obtained markedly different quantitative results. In rats receiving estradiol this ratio was computed to be 12:1 by Berger (1935) and 4:1 by Freud (1939). The latter author found a ratio of 2:1 for estrone, 1:1 for estradiol benzoate, and 1:12 for stilbestrol. Under somewhat different experimental conditions but using castrated rats also, Stadler and Lyons (1938) calculated a ratio of 50:1 for estrone and a ratio varying between 100:1 and 200:1 for estradiol benzoate. The latter hormone was found to be 30 times as potent as estrone when both were administered vaginally. The high degree of sensitivity to which this method of estrogenic assay can be brought is shown by the figures of Mühlbock (1940), who instilled the hormones in 50 per cent glycerine intravaginally in castrated mature mice. The effective estrogenic doses reported by Mühlbock were: for estrone, 0.00025 gamma; for estradiol, 0.0005 gamma; for estriol, 0.00075 gamma. The same order of sensitivity has been attained by Hartman and Littrell (1945) in a new test for blood estrogens. The test sample, in volume of 0.01 or 0.02 cc., is injected subcutaneously in immature rats in the region of the future vaginal orifice. A positive reaction consists of a transverse dimpling of the skin, usually within 24 hours, followed by the opening of the vagina within 4–5 days. In adapting this test to guinea-pig weanlings, Littrell, Tom and Hartman (1946) have been able to establish an exponential curve correlating the time of opening

of the vagina with the amount of estrogen injected locally into the perineum. When 20 gamma of estradiol dipropionate was injected vaginal opening occurred in 10 hours, while with a dose of 0.004 gamma opening was delayed to 96 hours.

Emmens (1941) has determined the relative minimal effective estrogenic dose by subcutaneous as compared with local (vaginal) administration (S/L ratio) for a large number of natural and synthetic estrogens as well as other compounds of questionable estrogenic status and has cleverly applied these data toward an analysis of the mode of action of estrogens. The compounds investigated by Emmens fell into 2 distinct groups on the basis of their S/L ratios; namely, those having a high S/L ratio, greater than 50, and those with a low S/L ratio, usually less than 2. The substances with a high S/L ratio, listed

TABLE 1

Substances having a high S/L ratio (Estrogens)

(after Emmens, 1941)

	S/L
Estrone	260
Estrone methyl ether	60
Estradiol	50
Ethinyl estradiol	120
Estriol	2000
Ethinyl-dihydro-equilin	90
Diethylstilbestrol	320
ψ-diethylstilbestrol	450
Diethylstilbestrol dimethyl ether	400
Ethyl-propyl-stilbestrol	170
Di-*iso*-propyl-stilbestrol	310
Di-*n*-butyl-stilbestrol	310
Hexestrol (meso)	180
Hexestrol (racemic)	360
4:4-Dihydroxy-γ:δ-diphenyl-β:δ-hexadiene	170
Triphenylchloroethylene	65
3:3′:4:4′-Tetrahydroxy-γ:δ-diphenyl-*n*-hexane	63
1-Ethyl-2(*p*-hydroxyphenyl)-6-hydroxy-1:2:3:4-tetrahydronaphthalene	ca. 125

in table 1, comprise the natural estrogens, many compounds of the stilbestrol series, hexestrol, and a few other synthetic estrogens. The substances with a low S/L ratio are listed in table 2. The sharp dividing line between these two groups results from the fact that the synthetic compounds listed in table 2 are scarcely if at all more active when applied directly to the vagina than when injected. Yet some of such synthetic compounds, after injection into female rabbits, result in the excretion of phenolic metabolic products which are more active estrogenically than the parent compounds (Stroud, 1940). Emmens concluded that these synthetic compounds are probably inactive themselves and must be transformed into active substances in the body. He therefore called them pro-estrogens. Their action upon the vagina was presumed to depend upon their systemic absorption, whether applied locally or by injection.

Thus the active metabolites were visualized returning to the vagina in no greater concentration or amount after vaginal than after subcutaneous administration.

In a later experiment Emmens (1942) proffered additional evidence that pro-estrogens are metabolized to estrogens in the body. Using the technique of Robson and Adler, he divided the vagina of spayed mice into 2 compartments, with the anterior one opening suprapubically and the posterior one retaining its normal perineal aperture. Estrogens, when applied locally to one sac, even in many times the minimal effective dose, produced a strictly local reaction without affecting the other sac. Pro-estrogens, on the other hand, stimulated cornification in both sacs when given in only minimal effective doses into one

TABLE 2

Substances having a low S/L ratio (Pro-estrogens)
(after Emmens, 1941)

	S/L
4-Hydroxydiphenyl	<12
4:4′-Dihydroxydiphenyl	<7
4-Hydroxydiphenyl ether	<7
4:4′-Dihydroxydiphenyl ether	<15
Stilbene	1.6
4-Hydroxystilbene	<1.0
4-4′-Dihydroxystilbene	<1.0
4-Hydroxy-α:β-diethylstilbene	<0.6
α-Phenyl-stilbestrol	1.5
α-Phenyl-β:β-di(*p*-hydroxyphenyl)-ethylene	0.4
Dihydroxyhexahydrochrysene	ca. 0.7
9:10-Dihydroxy-9:10-di-cyclopentyl-9:10-dihydro-1:2:5:6-dibenzanthracene	1.6
9:10-Dihydroxy-9:10-di-n-propyl-9:10-dihydro-1:2:5:6-dibenzanthracene	0.9
Dihydroxy-di-α-naphthyl-acenaphthene	ca. 0.33
p-Hydroxypropiophenone	ca. 4.0
p-Hydroxypropiophenone pinaeol	0.5
p-tert-Amyl-phenol	<2.0
4-Hydroxy-triphenylmethane	<3.0
trans-Androstenediol	0.9
Anhydro-hydroxy-progesterone	1.2
α-Ethylstilbestrol	<4.0

of them. It seems reasonable to conclude from these experiments that pro-estrogens do not become estrogenic until they have entered the systemic circulation.

The vaginal mucosa seems capable of hydrolyzing estrogenic esters with a high degree of efficiency. Emmens (1941) found that in instances where esterification of the true estrogens produced an increase in the S/L ratio, it did so by raising the minimal effective subcutaneous dose, while causing only small differences, on a molar basis, in the dose required vaginally. The effect of esterification on the S/L ratio is shown in table 3.

Clinical. Local hormonal therapy has found its greatest clinical usefulness in the treatment of vulvovaginitis, particularly in children. The histologic

character of the vaginal and vestibular mucosa of prepuberal girls and its concomitant low hydrogen ion concentration make this delicate membrane easy prey for the gonococcus as well as for non-specific bacterial invaders. After the discovery by Lewis in 1933 that systemic estrogenic therapy could produce hyperplasia of the vulvovaginal tissues and bring about clinical cure of gonococcal vulvovaginitis in young girls, several studies convincingly demonstrated that at least as high a degree of therapeutic success could be achieved by the vaginal administration of estrogenic hormone in gelatin capsules of Amniotin or suppositories of estradiol benzoate or stilbestrol (Te Linde and Brawner, 1935; Te Linde, 1938; Lewis and Weinstein, 1936; Lewis and Adler, 1936, 1937; Jacoby, Madonia, Till and Wood, 1939; Mazer and Shechter, 1939; Woodruff and Te Linde, 1942). Te Linde attributed the curative effects of this form of treatment to the increase in thickness of the vaginal mucosa, which was seen by biopsy; whereas Lewis and Weinstein, who discovered a reduction in vaginal pH follow-

TABLE 3

Effect of esterification on S/L ratio

(after Emmens, 1941)

	S/L
Estrone	260
Estrone butyrate	230
Estrone caproate	500
Estradiol	50
Estradiol benzoate	160
Diethylstilbestrol	320
Diethylstilbestrol dipropionate	190
Diethylstilbestrol dicaproate	300
Diethylstilbestrol dipalmitate	3000

ing local estrogenic treatment both in normal children and in others with vaginitis, regarded the increased acidity of the vaginal fluid as the important therapeutic factor in eradicating infection. Clinical cure occurred independently of systemic absorption of the hormone, for in only an occasional instance were side effects of the estrogen, such as slight mammary enlargement, observed. This contrasts with the relatively high incidence of precocious breast development, pubic hair growth, and uterine bleeding which occurred when estrogens were administered to children by hypodermic injection. Alternate methods of treatment for gonococcal vulvovaginitis, preferred now by most gynecologists, include the sulfonamides and penicillin.

Vaginal suppositories containing estrogens together with glucose and sodium phosphate (Kolpon Inserts, Roche-Organon) have also been used with success in the treatment of non-specific vaginitis in sexually mature women (Neugarten and Steinitz, 1941).

The atrophic changes which usually occur in the genital organs of postmenopausal women result in a lowered resistance of the vaginal epithelium to infection. Superficial ulceration and bacterial invasion are common. Sympto-

matic relief from the discharge, bleeding, itching, burning, soreness, and dyspareunia caused by senile vaginitis has been obtained by local treatment with Amniotin suppositories (Lewis and Adler, 1937) and with an ointment containing estriol, and the specific trophic effect on the vaginal mucosa has been demonstrated by biopsy (Mishell and Motyloff, 1940). Hamblen (1945) recommends the insertion nightly of a vaginal suppository containing 0.5 mgm. of diethylstilbestrol for 4–6 weeks, in order to avoid recurrences. The rationale for estrogenic therapy in patients with senile vaginitis is similar to that for vulvovaginitis in children. The results are similarly gratifying, but the relative importance of epithelial hyperplasia and the restoration of a low vaginal pH is still a matter of uncertainty.

Hamblen has also recommended prophylactic vaginal administration of diethylstilbestrol suppositories, both preoperatively and postoperatively, to postmenopausal women who require vaginal plastic operations. This treatment, it is claimed, together with application of diethylstilbestrol ointment to the vulva, renders the tissues less friable at the time of operation and favors per primum healing.

Uterus. *Experimental.* Cyclic hormonal control of the uterus is a biphasic phenomenon. A follicular, estrogenic, or proliferative phase of uterine development, characterized predominantly by endometrial growth and glandular proliferation, is associated with the follicular phase of the ovarian cycle and is reproducible in castrated animals by the injection of estrogenic hormones. A luteal, progestational, pregravid or (in primates) premenstrual phase, which occurs in association with the corpus luteum stage of the cycle, is marked by differentiation and secretory activity of the endometrial glands, and can be reproduced experimentally by the administration of progesterone to castrated animals which have been appropriately primed with estrogen. The biphasic nature of myometrial contractility has also been clearly related to hormonal control in certain species such as the rabbit, but in the human this relationship is still subject to differences of opinion and remains to be clarified.

The local action of estrogens on the uterus has been demonstrated in several species. Loewe and Voss (1926) produced localized hypertrophy of the guinea pig's uterus by injecting an estrogenic placental extract into a ligated segment of one uterine horn, using the other horn of the uterus as a control. This result was confirmed in the guinea pig and extended to young rabbits and castrated rats by Grumbrecht and Loeser (1938), using small doses of estrone, estradiol, and estradiol benzoate. The same authors introduced estrogen pellets into the uterus of amenorrheic women and demonstrated a proliferative effect on the endometrium by means of biopsies before and after treatment. Proliferation and hypertrophy of the endometrial cells are not inhibited by atropine; it is probable, therefore, that they represent specific effects of estrogen rather than the result of its vasodilating action (Hechter, Lev and Soskin, 1940). Perloff and Kurzrok (1941) succeeded in causing the formation of uterine fibromyomas in guinea pigs by the local implantation of 3 mg. pellets of estradiol into the uterus. This quantity of the hormone, however, might well have resulted in

tumor formation following systemic administration; tumorigenesis, therefore, cannot be attributed to its local action. In an *in vitro* experiment Gaillard and De Jongh (1938) failed to observe growth in thin slices of mouse uterus which were kept alive in a medium of plasma and salt solutions to which various concentrations of estrone were added. Using cover slip preparations of rat uterus Emmens and Ludford (1940) also found no response of the tissue to stilbestrol or to estradiol. In their hands, however, the vaginal tissues also failed to respond to the local action of estrogen by this technique.

McGinty, Anderson and McCullough (1938, 1939) were the first to demonstrate a local action of progesterone on the endometrium. These authors injected an oily solution of the crystalline hormone into a ligated uterine segment in immature rabbits which had been primed previously with estrogen. By this technique 0.5–5 gamma of progesterone was as effective in producing progestational transformation of the endometrium as was 0.5 mgm. of the hormone injected intramuscularly in divided doses over a period of 6 days; and threshold endometrial reactions were obtained with as little as 0.125 gamma of progesterone applied locally. These experiments have been confirmed in immature rabbits by Mussio-Fournier, Albrieux and Grosso (1939) and by Mennega and Tausk (1940), and in castrated rabbits by Sammartino and Nogués (1945).

The practical importance of the McGinty technique would seem to lie in its adaptability for the assay of blood or other biological fluids for their progestogenic activity. Results obtained by this method have been reasonably satisfactory from a qualitative standpoint, but the quantitative inaccuracies seem sufficiently great as to make the test unsatisfactory for better than very rough measurements of progesterone. Haskins (1939) obtained a positive test for progesterone with as little as 0.2 cc. of serum of a pregnant guinea pig. A smaller amount of serum failed to produce a progestational reaction in the rabbit's endometrium, and serum from a non-pregnant guinea pig was likewise ineffective. De Allende (1940) attempted to establish a curve of the normal progesterone content of the blood of the rhesus monkey during the menstrual cycle, by making assays of the serum of 3 animals every 3 days. While demonstrating progestogenic activity in the monkey's blood, her results failed to follow a consistent cyclic pattern. The amount of progesterone in the blood serum of the non-pregnant monkey was estimated to vary between a minimum of 0.06–0.12 gamma per cc. and a maximum of 0.25–2.5 gamma per cc. Using the same technique Haskins (1941) demonstrated progestational activity in 20 out of 21 samples of blood serum obtained from women at various stages of pregnancy up to 7 months. All the reactions in the rabbit's uterus were minimal, however, indicating, according to Haskins' calculations, that the serum probably contained less than 0.13 gamma of progesterone per cc.

Clinical. Karnaky (1942) has reported dramatic and immediate cessation of uterine contractions following the injection into the anterior lip of the cervix of large amounts of stilbestrol (25–100 mgm.) in oil in cases of threatened abortion and premature labor and has claimed excellent results with this method of treatment. There is considerable evidence that estrogens stimulate the pro-

duction of progesterone by the corpus luteum in the rabbit; and an increase in pregnanediol excretion has been observed following the treatment of a pregnant woman with stilbestrol (Smith, Smith and Hurwitz, 1946). So rapid was the therapeutic effect obtained by Karnaky, however, that it seems more likely that the administered medication acted directly on the uterus rather than through a metabolic process involving the mediation of progesterone. Whether the action was exerted primarily on the myometrium or whether primarily on a nerve plexus, whether it resulted from the estrogen or whether from the oil, are questions which remain to be answered. Hormonal injections into the cervix have not yet received general acceptance in the treatment of obstetrical complications (Hamblen, 1945).

Mamma. *Experimental. Nipple.* The nipple area is a specialized part of the integument which is particularly sensitive to hormonal stimulation. Tingling of the nipple, widening and hyperpigmentation of the areola, and hypertrophy of the tubercles of Montgomery are well known concomitants of pregnancy. Similar reactions may occur in non-pregnant women in response to appropriate endocrine therapy. In experiments on rats, mice, rabbits and guinea pigs, growth of the nipples is always observed when the animals are treated with adequate amounts of estrogenic hormone.

It has been a relatively simple matter to demonstrate the direct action of hormones, particularly estrogens, on the nipple and areola. By carefully regulating the dosage of the topically applied hormone, several experimenters have been able to produce unilateral growth of the treated nipple with little or no growth on the control side. Zondek (1935) was the first to show that percutaneous application of estrogen to the guinea pig nipple resulted in enlargement of this structure within 8 days. With the concentration of hormone used by Zondek, however, sufficient systemic absorption occurred so that soon the untreated nipple also began to enlarge and after 4 weeks there was no difference between the two. The guinea pig's nipple is remarkably sensitive to estrogenic stimulation. Several subsequent investigators have exploited this fact, using estrogens in a varicty of vehicles including several oils, ointment bases, and alcohol. Mussio-Fournier, Albrieux and Buno (1937) found that 25 I.U. of an estrogen applied locally to the base of the nipple of male guinea pigs daily for 15 days produced an increase in length of 124 per cent, as compared with an 81 per cent increase from the subcutaneous administration of 50 I.U. of the hormone daily for the same period of time. These results were abundantly confirmed in subsequent reports by Nelson (1941) and by Jadassohn and his co-workers, using various estrogens (Jadassohn, Uehlinger and Zürcher, 1937; Jadassohn, Uehlinger and Margot, 1938; Fierz, 1939; Jadassohn, Uehlinger and Fierz, 1941). Stilbestrol or estrone in a solution containing 1 gamma per cc., when applied to one nipple of male guinea pigs in the amount of 1 drop daily, caused in 30 days a tripling in the length of the treated nipple, a doubling of its width, hyperpigmentation of the nipple and areola, and an increase in the epithelial layer from a thickness of 2–4 cells to one containing approximately 30 layers of nuclei. Similar but less pronounced localized growth of the guinea-

pig nipple was obtained by topical application of testosterone propionate, androsterone, androstenedione, androstanedione, andrenosterone, and corticosterone. The pigmentary reaction in the guinea-pig nipple received special attention by Davis, Boynton, Ferguson and Rothman (1945). They found in castrated males that a local pigmentary response could be elicited by the topical application of diethylstilbestrol to the nipple area, thereby demonstrating that the action of estrogen on the melanoblasts is a direct one. A differential growth of the nipples of male rabbits was reported by Lyons and Sako (1940) and Lewis and Turner (1942) following unilateral local treatment with estrone or with diethylstilbestrol, the row of nipples receiving the estrogen growing at a faster rate than those on the control side. Similar results were obtained in toy fox terriers by Williams, Gardner and De Vita (1946) with estrone.

In the rhesus monkey, also, a topically applied solution of estrone resulted in unilateral nipple growth in young males (Speert, 1940; Chamberlin, Gardner and Allen, 1941). Painting one nipple of each of these animals daily with alcoholic solutions of estrone containing 0.05 mgm. per cc. and 0.5 mgm. per cubic centimeter for periods up to 75 days resulted in a 14-fold increase in the calculated volume of the nipple, while little or no growth occurred in the control nipple (Speert, 1948). After the cessation of treatment the experimental nipples involuted rapidly, but even 3 months later their average size was still 5 times as large as before treatment. A similar disparity occurred between the areolas on the treated and untreated sides. The same treatment of pregnant monkeys and a group of young females undergoing intensive systemic estrogenic therapy resulted in no demonstrable effect on the nipples or areolas. Failure to respond to an extraneous estrogenic stimulus is undoubtedly attributable to the state of estrogenic saturation which already existed in the tissues of these animals.

Supernumerary nipples are also sensitive to estrogenic stimulation, as is suggested clinically by their increased prominence and pigmentation in women during pregnancy. This suggestion was extended to the monkey by Zuckerman's observation (1935) of the changes in the supernumerary nipple of a male macaque following injection with estrogen. The topical application of estrone to a small supernumerary nipple in a young male monkey resulted in a 36-fold increase in its calculated volume, but identical treatment of a large accessory nipple in a mature female produced no change (Speert, 1942a; 1948). Here too the disparity in responsiveness of the supernumerary nipples can be ascribed to the differences in the growth stimuli to which they had already been subjected and their resultant varied capacity for further growth. This hypothesis will serve also to explain the failure of a supernumerary nipple in a recently pregnant guinea pig to increase in size after local treatment with estrone for 147 days (Speert, 1942b).

Mammary gland. The mammary gland proper, as well as the nipple structures, is capable of response to direct hormonal stimulation. Estrone in alcoholic or oily solution applied directly to the mammary area has resulted in glandular growth in male mice (Gardner and Chamberlin, 1941), castrated male and female guinea pigs (Nelson, 1941), male rabbits (Lyons and Sako,

1940; Lewis and Turner, 1942), and virgin heifers (Petersen, 1943). In virgin female goats inunction of the udder with estradiol benzoate produced complete mammary development, equivalent to that seen in the latter part of pregnancy (De Fremery, 1936), and similar treatment with an ointment containing 1 per cent diethylstilbestrol dipropionate resulted in copious lactation (Folley, Watson and Bottomley, 1940). In the latter experiments a daily yield of 1500 cc. of milk of normal composition was obtained, after a 30-day period of treatment which consisted of estrogenic inunction of the udder 3 times a week accompanied by daily milking. Since milking, combined with inunction of the udder with an ointment not containing estrogen, was also capable of initiating lactation, it is difficult to know how much to credit to the estrogen.

Unilateral growth of the mammary gland has been demonstrated in male monkeys after local treatment of one side with an alcoholic solution of estrone, the other side receiving alcohol alone as a control (Speert, 1940, 1948; Chamberlin, Gardner and Allen, 1941). In the monkey experiments the area of the estrogen treated mammary glands was greater than that of the controls and the duct system was better developed. Histologic sections revealed hyperplastic ducts and increased periductal connective tissue in the glands treated with estrone, in contrast to fewer, smaller, and more poorly developed ducts in the control glands.

These experiments have special significance because of their bearing on the problem of estrogenic control of the mammary gland. The mode of action by which estrogens produce their trophic effect on the mammary gland has been a matter of dispute for several years. Injected estrogenic hormones have commonly been found incapable of producing mammary development in hypophysectomized laboratory animals. This observation led to a large amount of experimental work and numerous publications, principally by Turner and his students, culminating in the theory that estrogens produce mammary development through the mediation of the anterior pituitary. It was postulated that this gland, under estrogenic stimulation, produces mammotrophic hormones called "mammogens", which in turn exert their trophic influence on the mammary gland (Gomez and Turner, 1938; Lewis and Turner, 1939; Mixner and Turner, 1943). Estrogens were held to be incapable of direct mammary stimulation. The evidence for and against this theory has been critically reviewed elsewhere (Speert, 1948). It will suffice here to point out that the demonstration in several species of unilateral mammary growth resulting from local application of estrogen cannot be reconciled with the mediation of this effect through the pituitary and is therefore regarded as critical evidence against this theory. Pituitary mediation, by requiring first systemic absorption of the estrogen, would be expected to result in bilateral, symmetrical mammary growth. It must be concluded, therefore, that estrogens can act directly on the mammary gland.

A report by Lyons (1942) has indicated that lactogenic hormone can also act locally upon the mammary gland. When this substance was injected intraductally, through the nipples of rabbits, localized lactation ensued in the injected parts of the gland while the control sectors remained unaffected.

Earlier studies had shown that the sensitivity of the crop-sac method of assay for lactogenic hormone could be greatly increased by the intradermal injection of the substance over the crop-sac. This method of administration permitted the detection of the lactogenic hormone in a dose of 1 gamma, in contrast to 100 gamma required by the intramuscular injection (Lyons and Page, 1935; Bates and Riddle, 1936). An improved method of preparation allowed for a positive local response with only 0.1 gamma (Lyons, 1937a). By injecting the hormone locally in divided doses over a period of 4 days Lyons (1937b) attained a further ten-fold increase in sensitivity. When lactogenic hormone is injected intradermally over the crop-sac of the pigeon the histological effects of stimulation are limited to the area immediately subjacent to the site of injection (Lyons, 1937a), the rest of the crop-sac wall being unaffected.

Clinical. Gratifying but temporary cosmetic improvement has been obtained in cases of hypomastia treated with estrogenic hormones. This condition is frequently associated with general sexual hypoplasia; in some cases, however, genital structure and function are normal and the developmental defect appears to be localized in the breasts. In the treatment of patients in the latter category it is important not to disturb normal ovarian function by systemic hormonal therapy. Local treatment with estrogenic ointment provides a means of concentrating effective amounts of the hormone in the breast with minimal effect on the other organs of the body. MacBryde (1939) treated a small series of patients with generalized sexual underdevelopment and hypoplastic breasts by daily inunction of one breast with 5 grams of an ointment containing 5,000 I.U. of estradiol or estradiol benzoate per gram. The treated breast enlarged more rapidly than that on the untreated side, although sufficient systemic absorption occurred to produce enlargment of the uterus and changes in the vaginal mucosa. Good results with this method of treatment were also reported by Cernea (1940), who had his patients rub 5 grams of an ointment containing 0.1 per cent stilbestrol into each breast nightly. Mammary enlargement was obtained in 30 days but treatment had to be repeated from time to time.

Ointments containing therapeutically effective concentrations of estrogen must be differentiated from fraudulent products which occasionally appear on the market and are advertised to the public as "bust developers" (J. A. M. A., 1940, 1943, 1944).

Puerperal mastitis has been treated by local inunction of the affected breast with stilbestrol salve and Leinzinger and Bayer (1940) claim excellent results with this method of treatment. In a series of 64 cases of acute puerperal mastitis suppuration occurred only twice, resulting in 90.6 per cent cure attributable to the stilbestrol inunctions, as compared with 71.4 per cent cure attained in a control series which received x-ray therapy. Local androgenic treatment in the form of testosterone or testosterone propionate inunctions was used by Spence (1940) for the relief of pain in cases of so-called chronic mastitis. Mammary pain was reported alleviated in 8 out of 10 patients so treated. The rationale of such types of therapy for either inflammatory or proliferative mastopathies remains so obscure that acceptance of hormonal inunctions as a treatment of choice must await confirmatory reports by other observers. Dunn (1944) has reported

negative results in the local as well as the systemic treatment of gynecomastia with androgenic hormone. Local treatment consisted of the injection into the substance of the affected breast of 50 mg. of testosterone propionate in 1 cc. of sesame oil, such injections being repeated at weekly or bi-weekly intervals for a total of 200 mgm. of the hormone.

Vulva. Perineal itching or pruritus vulvae is a rather common affliction of elderly women. Success has been reported by Klaften (1937), Zondek (1938), and Lubowe (1941) in treating this condition by the local application of ointments containing natural or synthetic estrogen. Sustained symptomatic relief, however, usually requires prolonged or repeated treatment, the safety of which has been challenged because of the danger of carcinogenesis. For the same reason even stronger objection has been raised against the use of estrogens in the treatment of krausosis vulvae, an atrophic lesion of the vulvar tissues with which pruritus is usually associated. Clinical cure of patients with this disease by local treatment with estrogenic ointment has been reported in a few instances (Jaffé, 1937; Tscherne, 1938) and the proliferative changes induced in the atrophic skin of the vulva have been demonstrated by biopsies before and after treatment. After many years of trial, however, Frank (1940) has found estrogenic therapy for kraurosis vulvae to be purely palliative and the improvement transient. Because of the high incidence of malignancy which results from the leukoplakic changes frequently associated with kraurosis, most gynecologists now regard excision or vulvectomy rather than hormonal therapy as the treatment of choice.

Skin. A variety of dermatologic disorders involving other parts of the body's surface have been treated topically with hormones. In reviewing the results of treatment of skin affections, it is important to remember that here perhaps to a greater extent than in most of the other clinical entities reviewed, spontaneous improvement is common, psychologic factors are often of great importance, and that a tendency exists among clinicians to report favorable experiences with new therapeutic methods to the exclusion of equivocal or frankly negative results.

Experimental. In a series of old rats which were losing their hair and in another group of infantile rats with alopecia, Kun (1937) found estrogens to be effective in causing hair growth. After rubbing an ointment containing estradiol benzoate into the hairless parts of these animals for 1–2 months, complete growth resulted, while the control rats showed no change. A direct effect of the hormone on the hair follicles is suggested by the observation that growth occurred first at the sites where the ointment was applied. In apparent contrast to these findings is the recent report of Williams, Gardner and De Vita (1946). These authors observed a distinct inhibition of the regrowth of hair in three toy fox terriers after a solution of estrone was applied locally to shaved areas of the lateral body walls, whereas control areas which were treated with the solvent alone showed a normal rate of regrowth.

Estrogens are capable of increasing the water content of the skin. Selye (1944) treated the so-called hairless mouse, whose skin is affected by a congenital

anomaly, with topical applications of a solution of estradiol containing 250 gamma of the hormone per cubic centimeter. The treated animals responded with a non-pitting cutaneous edema, similar histologically to that which occurs in the sexual skin of monkeys. This phenomenon is probably similar to the premenstrual edema which develops in many normal women and the physiological edema of pregnancy, both of which are believed to be estrogenic effects. Another effect of estrogens in the rhesus monkey consists of reddening of the sex skin. Chamberlin, Gardner and Allen (1941) produced such reddening unilaterally by the local application of an alcoholic solution of estrone.

Plumage pigmentation in birds is intimately dependent upon the sex hormones. In the brown Leghorn, for example, the breast feathers are normally light colored in the hen and black in the capon. If a capon is treated with estrogen while its feathers are growing, a light brown bar makes its appearance in each feather, the width of the bar corresponding to the duration of estrogenic activity. Greenwood and Blyth (1935b) found that the local intradermal injection of small amounts of estrone, too small to cause a plumage response when injected into the pectoral muscles, induced local female pigmentary changes, the replacement of black by red, in the breast feathers nearest the point of injection. 'Espinasse (1939), who confirmed these observations, noted that the side of the feather toward the site of injection tended to react more and sooner than the distal side. Deanesly and Parkes (1937) obtained similar results after the inunction of one feather tract with estradiol and estradiol benzoate.

The direct action of sex hormones on the differentiation of melanophores in birds was demonstrated by Hamilton (1940) by means of tissue culture experiments. He explanted bits of dorsal skin from 6-day-old New Hampshire Red and Rhode Island Red chick embryos. Differentiation of red melanophores and increased production of pigment were stimulated by estrone, estradiol benzoate, estradiol dipropionate, and testosterone propionate, which were added to the culture medium in concentrations between 20 gamma and 300 gamma per cc.

Clinical. Increased pigmentation of the nipples, areolas, and linea alba occurs during pregnancy and can be produced in non-pregnant women and in experimental animals by treatment with large amounts of estrogen. Davis, Boynton, Ferguson, and Rothman (1945) demonstrated a direct stimulating action of estrogen on melanoblasts. It is therefore difficult to understand with what rationale Rocca (1942) treated his patient, although of menopausal age, with estrogenic hormone for pigmentary patches on her face. Yet after combined treatment with injections and local inunctions of estradiol for several months the facial pigmentation diminished and Rocca attributed the happy result, perhaps gratuitously, to the hormone.

In a better controlled series of observations, consisting of biopsies before and after treatment, Goldzieher (1946) observed a trophic effect of estradiol ointment and diethylstilbestrol ointment on the skin of the forearm of five elderly women. His published photographs of sections of skin obtained after six weeks of daily inunction with these hormones show distinct regeneration of the previously atrophic epidermis.

Testosterone propionate has been used with success in the treatment of senile dermatoses in men, but in the reported cases the results cannot be attributed to a local action of the hormone on the skin. In a series of Holander and Vogel (1942) improvement was obtained in 8 cases after testosterone ointment was applied to the *unaffected* parts of the skin; and in the patients treated by Dobes, Jones, and Franks (1945) for pruritus, injections of the hormone were given simultaneously with the inunctions.

Jaffé (1937) and Zondek (1938), the latter a pioneer in so many of the new uses for estrogens, claimed a salutary effect of estrogenic ointment on acne vulgaris in young girls and in menopausal women. This treatment was studied more critically in a well controlled series of cases by Lynch (1941), who found no better results among the girls using an ointment containing 4,000–5,000 R.U. of estradiol per ounce than in the control patients who used the ointment base alone. Lynch emphasized the natural tendency of acne to improve when treatment consists of only soap and water. Jaffé also reported beneficial results from percutaneously administered estrogen in the treatment of seborrheic dermatitis and a case of chronic leg ulcer with surrounding eczema.

Hormones have been used locally in attempts to treat ringworm of the scalp, since they have been found to possess fungistatic activity in vitro; but Lewis, Hopper and Reiss (1946) reported disappointing results after applying diethylstilbestrol and testosterone ointments locally to the areas of infection.

Estrogens have been incorporated in some commercial cosmetics, the advertising of which has often been unrestrained and the public exploited to the limit of its gullibility. An editorial in *The Journal of the American Medical Association* (1938) quoted the following advertising copy, which bore the approval of Good Housekeeping Bureau: "Endocreme contains a counterpart of the natural bodily element which helps account for that pink freshness of a youthful skin. The substance is known to the medical world as es-tra-diol, and Endocreme is the only cosmetic which contains es-tra-diol. It helps replenish the supply of the very substance which your skin may lack! Endocreme, therefore, is more than 'just a face cream'. It's an active, vital cosmetic which begins to work with the substructure of the skin. In later life the skin tends to fall into lines and wrinkles because the tissues underneath the skin sag. When Endocreme firms and tones these underlying tissues, the skin is smoothed out again . . . pores decrease in size . . . and lines begin to disappear." According to the manufacturer's claim, Endocreme contained 0.625 mgm. of estradiol per ounce, and the purchaser was instructed to apply one-half teaspoonful to the face, neck, arms and hands every night.

As recently as May 12, 1946 The New York Times carried the following advertisement: "Your birthday confidante . . . Gourielli's 'Look Younger' Cream ESTROLAR. Trust Gourielli Estrolar *implicitly* to keep each birthday a secret . . . to deny your age discreetly . . . defy it to show on your face. You *can* . . . for this 'Look Younger' Cream contains an Estrogenic Complex . . . the equivalent of a precious body substance which is abundant in youth but diminishes with every passing year. This Estrogenic Complex is an important aid in attain-

ing a younger appearance of face, throat and hands. 30-day supply, 5.50. Twin jars, 10.00". This cream is still being advertised.

Protection of the public from similar products will result from orders such as that recently issued by the Louisiana State Board of Health (J. A. M. A., 1946a): "The manufacture, processing, packing, sale or distribution of any cosmetic or beauty preparation containing estrogenic hormone, any of its chemical derivatives or any synthetic chemical products possessing properties similar to estrogenic hormone is hereby prohibited in Louisiana."[1]

PENIS. In a letter to the editor of *The Journal of the American Medical Association* published August 9, 1941 Schiller referred to a novel "The Golden Lotus" by Wang Shih-Cheng (d. 1593) which described Chinese life and customs, including some details of medical practice, during the Sung dynasty in the reign of Hui Tsung (1100–1126). Quotations from the English and German translations of this novel indicated that modern therapy based on the local application of sex hormones had its counterpart in oriental medicine centuries ago. An exchange of gifts is related between a Hindu monk and a wealthy pharmacist in which the latter was presented with "a little box with a red ointment, a little more than one-fifth of an ounce to anoint the glans penis, sufficient for one hundred and ten applications." The androgenic properties of the remedy were described as follows: "New strength will be given to limbs and belly, it will refresh the testicles, invigorate the penis . . . in a hundred days, hair and beard will be black once more, in a thousand days, your body will know its power. Your teeth will be stronger, your eyes more bright, your manhood made rigid . . . .".

In a modern experiment by Wigodsky and Greene (1940) it was found that 7.5 gamma of testosterone, when applied daily in alcohol or an ointment to the penis of castrated immature male rats, caused distinct growth of that organ in 10–22 days; estrone and estradiol, on the other hand, were without effect. Schiller mentioned similar treatment of human patients, designed to produce more frequent and more persistent erections, and local estrogenic applications to the clitoris of frigid women, but the results of these experiments have not yet been published.

SEMINAL VESICLE. Androgens can act directly on the epithelium of the seminal vesicle. This has been demonstrated in two independent experiments on rats. Lacassagne and Raynaud (1937) found that when testosterone propionate is injected into the interior of the seminal vesicle of a castrated animal, the vesicular epithelium can indicate the presence of as little as 2.5 gamma of the hormone. This is less than the amount required when testosterone propionate is administered by any other channel. Cells of the rat's seminal vesicle, which were grown in tissue culture by Demuth (1940), were seen to enlarge following the addition of testosterone to the culture medium.

[1] This regulation was later modified as follows: "No cosmetic or beauty preparation containing as one of its ingredients estrogenic hormone, any of its chemical derivatives or any synthetic chemical product may be manufactured, processed, packed, sold or distributed in Louisiana unless its label bears adequate directions for use and its label bears the number of international units per ounce of such ingredient" (J. A. M. A., 1946b).

CHICK'S COMB. The male sex hormones cause growth of the chick's comb. When androgens, either pure or in urinary extracts, are injected into chicks of either sex, stimulation of the comb occurs. For assay purposes capons or young cockerels are usually used. Fussgänger discovered in 1934 that the sensitivity of this test for androgens could be increased 50 times if the hormone was applied directly to the comb with a paint brush instead of being injected elsewhere into the body. If the androgenic substance was applied percutaneously to the back, however, it was only $\frac{1}{150}$ as effectual as when applied directly to the comb. Dessau (1937) also found that only $\frac{1}{150}$ as much male urinary extract was required for a positive response when smeared on the capon's comb as when the same extract was injected intramuscularly. The increased effectiveness of androgens administered locally to the capon's comb was confirmed for androsterone by Greenwood and Blyth (1935b), who demonstrated a greater response when the hormone was injected into the comb than when the same dose was injected into the pectoral region; and for testosterone and testosterone propionate by Deanesly and Parkes (1937), who produced a 200-fold increase in effectiveness by applying the hormones to the comb by inunction in propylene glycol. Ruzicka (1935) found that as little as 1–2 gamma of androsterone daily, applied locally in oil, could produce great growth in the comb of either sex.

Subsequent workers discovered that the sensitivity of the test could be increased further by appropriate choice of a vehicle. For example, alcohol was shown to be superior to sesame oil (Klempner, Frank and Hollander, 1940). The volume in which the androgen was dissolved was also important. The smaller the volume, and hence the greater the concentration, the greater the response in comb growth (Klempner, Hollander and Frank, 1940). In ascertaining the amount of growth some investigators have relied on the comb's weight (Danby, 1938; Frank and Klempner, 1937) after sacrificing the chicks; while others, using capons, have resorted to measurements of comb area (Oesting and Webster, 1938). Ruzicka (1935) observed an increase from 400 sq. mm. to 2700 sq. mm. in the combs of capons treated locally with an oily solution of 1 per cent androsterone for a week. After the cessation of treatment the comb usually continues to grow for about a week, then gradually returns to its normal size during the next 2–3 months.

Androgens to which the capon's comb has been found responsive after local treatment include testosterone, testosterone acetate, testosterone propionate, methyl testosterone, androsterone, androstenedione, adrostanedione, dehydroandrosterone, androstanediol and iso-androstanediol. In contrast to the different potencies which the various androgens have when injected intramuscularly, androstenedione, androstanedione, androstanediol, and androsterone were found to be about equally effective when applied directly to the comb (Dessau, 1937). A general correlation exists between the dose of the locally applied androgen and the percentage increase in the size of the comb (Dessau, 1937). Not all authors are in agreement, however, concerning the adequacy of this method for the assay of androgens. McCullagh and Guillet (1941), for example, regarded the inunction of the capon's comb as the most satisfactory of androgenic assay

methods, but admitted that the results were only roughly quantitative at best. Hoskins, Beach, Coffman and Koch (1941), who injected oily solutions of testosterone propionate into the comb of day-old cockerels, concluded that this method was of doubtful value for assay purposes. A more frankly pessimistic view of the problem was taken by Duff and Darby (1941). These workers found that the variation in response of the comb of day-old and 7-day-old chicks to androgens, either locally by inunction or by injection, was so large as to make quantitative assay impossible.

Nevertheless, despite its shortcomings as a quantitative test, the comb test is apparently quite specific for androgens. No response occurred to estrone, estriol, estradiol (Parkes, 1937) or estradiol benzoate (Frank and Klempner, 1937). The local application of progesterone, even in one hundred times the effective dose of androsterone, was without effect on the capon's comb (Dessau, 1937). On the contrary, Mühlbock (1938) found that the addition of estrogen or progesterone to testosterone ointment inhibited the action of the latter on the cock's comb. Inhibition of the capon comb's response to androsterone, whether administered locally or intramuscularly, was produced by estrone, estradiol, and diethylstilbestrol (Emmens and Bradshaw, 1939). The estrogens were more effective when applied directly to the comb. The ratio of the dose of estrogen required by inunction to the dose required by injection to produce this inhibition was 1:470 for estrone and 1:180 for estradiol, but only 1:2 for diethylstilbestrol. The stimulating activity which was demonstrated in extracts of pigs' ovaries applied locally to the capon's comb apparently resulted from an unidentified androgenic substance in the former (Parkes, 1937).

SPARROW'S BILL. One of the distinguishing sexual characteristics of the male English sparrow (Passer domesticus) is the black pigmentation in its bill. This pigmentation can be produced in the bills of female birds by treatment with androgens and seems to represent a specific effect of the male hormone. When the hormone is injected, however, the series of color changes is such as to make recognition of the end point difficult. Seeking a specific and quantitative assay method for androgens, Kirschbaum and Pfeiffer (1941) applied testosterone propionate locally to the angle of one side of the bill of sexually inactive female sparrows. Two gamma of the hormone administered daily in this manner resulted in a heavy deposition of melanin within 10 days on the treated side but none on the untreated side. In later and more extensive experiments by the same workers (Pfeiffer, Hooker and Kirschbaum, 1944), castrated males were found to be more uniform and more sensitive in their pigmentary response to locally applied androgen. One alcoholic drop containing as little as 0.063 gamma of testosterone, applied daily to the skin-bill junction, was capable of producing a unilateral pigment line in the bill. By systemic administration, on the other hand, 8 gamma of the hormone was required daily in order to elicit a pigmentary response, and this was bilateral and diffuse.

Testosterone and androsterone were about equally potent in their effect upon the sparrow's bill. Neither estrogens, progesterone, nor desoxycorticosterone acetate, however, was capable of producing pigmentation. Estradiol benzoate,

even in the relatively large dose of 10 gamma daily, failed to modify the bill's response to 0.25 gamma of testosterone when both were applied locally. This contrasts with the inhibitory effect of estrogen on the response of the capon's comb to androgen.

Because of its simplicity, specificity, low cost, and greater sensitivity, Pfeiffer, Hooker, and Kirschbaum regard this test as superior to the chick's comb for the quantitative assay of androgens. Values obtained by them in assaying crude extracts of testicular tissues of pigs and bulls by the two methods were in good agreement.

NASAL MUCOSA. *Experimental.* The existence of a naso-genital relationship has been suspected for over half a century. Recent physiologic observations afford some basis for the presumed clinical relationship between the nasal mucosa and genital function. Some of these experimental findings have been reviewed by Rosen (1942), who was able to demonstrate an uninterrupted nervous pathway from the nasal mucosa to the anterior pituitary, via the sphenopalatine and Vidian ganglia. Pseudopregnancy, it was learned, could be produced in the rat by excision of the sphenopalatine ganglion, which interrupted the non-olfactory innervation of the nose. The nasal mucosa has been associated with ovarian function in the rhesus monkey by the observation of Mortimer, Wright, and Collip (1936) that peaks of nasal activity, vascular in nature and consisting of reddening and swelling of the conchae, occur predominantly in menstruating monkeys, usually during the premenstruum. In order to determine the effect of estrogens on ciliated mucosa, Boyd, Clark, and Perry (1941) applied various concentrations of them topically to the buccoesophageal mucosa of the frog. Stimulation of ciliary movements was produced by solutions containing 0.07 – 0.2 mgm. of estrone per 100 cc. of saline, 0.002 – 0.02 mgm. of estriol per 100 cc. and 0.00005 – 0.0002 mgm of estradiol per 100 cc. Higher concentrations exerted an inhibitory effect on the cilia. These reactions occurred independently of the blood supply, for the mucosa was examined after being stripped from the frog's body.

Clinical. Following their observations on monkeys Mortimer, Wright, and Collip (1937) were led to attempt the treatment of patients with atrophic rhinitis by means of locally applied estrogen. After spraying oily solutions of estrone and estradiol (0.1 mgm. per cc.) into each nostril twice daily, 31 out of 38 patients experienced a significant degree of improvement in 2 – 6 weeks. Using similar treatment, coupled sometimes with local swabbing or packing of the nose with cotton pledgets dipped in estrogenic solution, Blaisdell (1938), Eagle, Baker, and Hamblen (1939), and Safer (1942) also reported good results in the treatment of atrophic rhinitis. Biopsy specimens, however, which Eagle, Baker, and Hamblen obtained from the middle turbinates in several cases before and after treatment, failed to reveal any consistent histological changes.

The same technique of treatment was used later by Mortimer, Wright, Thomson, and Collip (1939) for patients with so-called constitutional deafness. The daily nasal insufflation of 1 cc. of oil containing 1000 I.U. of estrogen was reported to result in "marked improvement of the aural defect and significant amelioration of the hearing level" in certain patients with this disease.

Multiple benign papillomata of the larynx occur typically in young children and tend to stop growing at puberty. Suspecting a relationship between this alteration in the course of the disease and the laryngeal changes which occur under gonadal influence, and stimulated by the success of Te Linde and Brawner in their treatment of gonococcal vaginitis by the direct application of estrogen to the vaginal mucosa, Broyles (1940) attempted to treat laryngeal papillomas in children by applying the hormone directly to the larynx. Whereas other forms of therapy for this condition have been unsatisfactory, Broyles reported distinct improvement, with regression of the growths, in 6 cases. Treatment consisted of a weekly spraying of the larynx, through a direct laryngoscope, with about 0.1 cc. of an oily solution of Amniotin containing 10,000 I. U. per cc.

COMMENT

There is abundant evidence in various species of animals and in man that the steroid and synthetic sex hormones can be absorbed in functional quantities from several of the body surfaces. To illustrate, growth of the prostate and seminal vesicles resulted from the application of testosterone to the penis of the rat (Wigodsky and Greene, 1940). Pratt and Smeltzer (1929) described the nasal absorption of estrogen in the human and the nasal and conjunctival absorption of estrogen in the rat. Experiments on the absorption of steroid hormones from the mucous membranes of the mouth were reviewed in a recent paper by Corner (1944). Vaginal absorption of estrogen, sufficient to induce menstruation in castrated monkeys, was reported by Powers, Varley and Morrell (1929).

Most impressive, both because of their large number and variety and because of their practical bearing on human therapeutics, are the experiments which demonstrated the percutaneous absorption of the sex hormones. Estrone applied to the skin in an oily vehicle caused vaginal estrus in spayed albino rats (Baer, 1939) and mice (Zondek, 1938). When the hormone was dissolved in benzol, ether, or alcohol it was just as effective administered percutaneously as when injected. Burrows (1945) has found that one drop of an estrogenic solution, applied locally to the skin of the interscapular region of mice twice a week, produces all the recognized estrogenic effects in the treated animals, including mammary cancer.

The androgen "Enarmon", rubbed into the skin of castrated immature male rats, caused development of the accessory sex organs (Ito, Hajazu and Kon, 1937). Sexual receptivity was induced in castrated female guinea pigs by the percutaneous administration of estrogens followed by progestin (Leighty, Wick and Jeffries, 1940). In males of the same species Moore, Lamar, and Beck (1938) produced growth of the nipples by rubbing an estradiol cream into the dorsal skin of shaved animals, and Ruinen (1932) produced mammary growth in rats, even in hypophysectomized males, by rubbing estrogen into the ventral skin. In a later report Zondek (1941), using estrogens percutaneously again, confirmed the observation of Bloch and Schrafl (1932) that these hormones increased the pigmentation and resulted in a strongly positive "dopa" reaction in the guinea pig's nipple and areola. According to an editorial in The Lancet (1938) a group of males employed in packing ampules of estrogenic material developed

tender swelling of the breasts as a result of handling the estrogenic compound. More recently 28 additional cases of frank gynecomastia, confirmed in 3 by histological examination, were reported among a group of 38 men engaged in the manufacture of stilbestrol (Fitzsimons, 1944). In the experiments of Ito, Hajazu, and Kon an estrogenic cream rubbed into the skin of infantile female monkeys caused within 2 weeks reddening and swelling of the sex skin, enlargement of the nipples, and an increase in uterine size. Loeser (1937) reported turgescence of the perineal region of castrated baboons and enlargement of the breasts of castrated and amenorrheic women after rubbing estradiol benzoate into the skin. He also found it possible to relieve menopausal flashes in 2 patients by this method of giving estrogen. Salmon (1938) observed full estrogenic changes in the vaginal smears of patients with little or no ovarian function, following the nightly inunction of their skin with an ointment containing estradiol. So effective is percutaneous absorption of estrogens in the human that Zondek (1938) reported satisfactory results in the treatment of menopausal arthritis as well as cases of primary and secondary amenorrhea with an estrogenic ointment.

The literature cited in this review has shown that localized tissue response to the sex hormones can be elicited by the direct application of these compounds to some of the body surfaces and internal organs. The concentration and amount of hormone required for the production of its effect locally without at the same time allowing significant systemic absorption, necessitates careful regulation such as is usually possible only in laboratory animals, several of which can be used in an experiment and be subjected to different doses. The fine adjustment of dosage necessary to permit a local action without systemic effects cannot be achieved consistently in the treatment of human patients. In attempting local therapy with either estrogens or androgens one must accept the fact that the amount of hormone applied, if it is to accomplish the desired local effect, will probably result in systemic absorption also. What, then, are the risks associated with the systemic absorption of these hormones?

Clinical experience has shown that adequate local estrogenic treatment for vulvovaginitis can be given to young girls with a relatively low incidence of undesired side effects. Slight mammary enlargement and occasional uterine bleeding, when they do occur, can reasonably be regarded as mild and transient complications. Their importance lies primarily in their psychological effect. The prescription of prolonged or repeated courses of estrogenic inunctions for mature women requires more circumspection. The continuous administration of estrogens interferes with normal ovarian function. In a large proportion of women with inadequate breast development, for whom estrogenic inunctions seem indicated for cosmetic reasons, ovarian function is already at a low level, making this consideration unimportant. In others, however, in whom the mammary defect exists despite the presence of normally functioning ovaries, a depression of the latter is liable to result from treatment adequate for breast development. As in the various species of animals, even among different strains of the same species, so in the human being the mammary tissue of different indi-

viduals varies in its sensitivity to estrogenic stimulation. If normal ovarian activity has failed to produce adequate mammary development, large amounts of estrogen would seem to be required for a reasonable expectation of a favorable response. In women of the childbearing age the possible impairment of their fertility, even though it be temporary, might well be a more important consideration than an augmented bust measurement, and hence be an effective deterrent against estrogenic therapy.

A stronger objection against prolonged estrogenic treatment is based upon its possible relation to carcinogenesis. In mice, and to a lesser degree in a few other species, hormonal factors are of fundamental importance in the production of cancer, especially mammary cancer. Castration dramatically reduces the incidence of mammary cancer in females of a susceptible strain (Lathrop and Loeb, 1916); and estrogenic treatment of male mice, which normally do not get the disease, results in tumor formation in them (Lacassagne, 1932). Uterine cancers have also been produced in experimental animals by means of estrogens (review by Greene and Brewer, 1941).

The relationship between cancer and estrogens is by no means as clearly established for man as for mice. Suspicion is aroused, however, by case reports like those of Allaben and Owen (1939), Auchincloss and Haagensen (1940), and Parsons and McCall (1941), in which breast cancer developed in women who had been receiving estrogenic hormone for 1–4 years; and those of Henry (1945) in which a definite adenocarcinoma of the endometrium was discovered in one patient and a "precancerous" endometrial hyperplasia in another, after 3 years of treatment with stilbestrol and estradiol, respectively. Fremont-Smith, Meigs, Graham and Gilbert (1946) have recently reported another case in which endometrial cancer appeared in a patient during a prolonged period of estrogenic therapy. The reviewer has personal records of several similar cases. The observations of Herrell (1937) have also suggested the importance of the continuous action of endogenous estrogens for the development of carcinoma of the endometrium, but Taylor and Millen (1938) indicated that there is reason for questioning this concept. Mention has already been made of the possible danger of estrogenic treatment of kraurosis and leukoplakia vulvae.

In the absence of more precise knowledge on this subject, a properly conservative attitude demands that prolonged estrogenic therapy, either local or systemic, be withheld from patients with a personal history of cancer of the breast or uterus, with a strong familial disposition to cancer, or with a precancerous genital lesion. The same type of reasoning leads to the conclusion that, until it can be definitely shown that they have no relation to the development of prostatic tumors, androgens should be administered to men with caution and for short periods of time, whether by injection or by inunction.

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PTEROYLGLUTAMIC ACID AND RELATED COMPOUNDS

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IT IS A COMMON OBSERVATION that the interpretation of scientific studies often becomes possible in the light of knowledge that is subsequently acquired. This observation has a strong application to an evaluation of the early work in the field of nutritional deficiencies. Descriptions of the specific lesions arising from the lack of a vitamin have commonly preceded alike the experimental definition of the uncomplicated deficiency and the chemical understanding of the missing nutrient. Particularly is this to be noted in the case of the vitamin B factors. The recognition and identification of the "newer" members of the vitamin B complex was preceded by early work which described nutritional lesions. With the progress of knowledge, more complete basal diets became possible as one by one the B vitamins were made available in pure form. In the case of pteroylglutamic acid, the interpretation of biological results was complicated by the existence of several naturally occurring derivatives of a parent molecule and by the variation which was encountered among different species of test organisms in response to these derivatives.

The biological effects of pteroylglutamic acid have been observed under a variety of circumstances which have given rise to a number of different names for the vitamin. The more commonly used names are listed in table 1. The name "folic acid" is widely used, doubtless due to its brevity and euphony, but since this name refers to an unidentified factor which was measured with *Streptococcus lactis* R, it is preferable to adopt the somewhat more cumbersome but chemically defined term, pteroylglutamic acid. Not included in table 1 are two conjugates which have been isolated in pure form; "fermentation *L. casei* factor" and "vitamin B_c conjugate". For "fermentation *L. casei* factor" (1) the name pteroyltriglutamic acid has been used (2), and in keeping with this nomenclature we have used the term pteroylheptaglutamic acid in preference to the longer name pteroylhexaglutamylglutamic acid (3) for "vitamin B_c conjugate".

The complexity of the nutritional relationships of the pteroylglutamic acid "family" of compounds has been to some extent lessened by recent studies in the fields of enzymology and structural chemistry. The following observations bear on the subject.

1. The parent molecule, pteroylglutamic acid, consists of a pteridine grouping linked through para-aminobenzoic acid to a single glutamic acid residue (fig. 1).

2. Successive molecules of glutamic acid may be attached in peptide linkage to the first glutamic acid radicle. Compounds with respectively three and seven glutamic acid groups have been isolated. These compounds have been termed "conjugates".

3. The growth of the test organism *S. fecalis* R is not markedly promoted by either of the two conjugates; the conjugate with seven glutamic acid groupings does not appreciably stimulate the growth of *Lactobacillus casei*.

4. Certain animals, including chicks, rats and, probably, monkeys, can utilize the conjugates as sources of pteroylglutamic acid when fed.

5. The conjugates are split with the liberation of pteroylglutamic acid by specific enzyme systems which are present in mammalian liver and kidney, avian pancreas, and other animal tissues. Such a system is also present in

TABLE 1

Nomenclature of preparations having biological activity presumably due to pteroylglutamic acid

NAME	DESCRIPTION	DATE	REFERENCE
None	Yeast extract, effective in the treatment tropical macrocytic anemia	1931	(4)
Vitamin M	Yeast and liver extract, effective against nutritional cytopenia in monkeys	1938	(5)
Factor U	Yeast extract, promoted growth in chicks	1938	(6)
Vitamin B_c	Adsorbed on fullers' earth, prevented nutritional anemia in chicks	1939	(7)
Norite eluate factor	From yeast and liver, active for *L. casei*	1940	(8)
Folic acid	Active for *S. lactis* R; concentrates prepared from spinach	1941	(9)
L. casei factor	Isolated from liver and yeast	1943	(10)

In addition to the above, less clearly defined fractions have received separate names, including vitamins B_{10} and B_{11} (11) and factors R and S (12).

certain vegetable materials, including almonds and potatoes, but is perhaps absent from *S. fecalis* R. These observations are thought to account for the finding that certain preparations were active when fed to deficient animals but were relatively inert in the microbiological assay. Treatment of such preparations with the specific enzyme system usually increases their potency in the microbiological assay.

6. The erythrocyte maturation factor of U.S.P. concentrated liver extracts is not identical with pteroylglutamic acid, although the factor and pteroylglutamic acid both produce identical hemopoietic responses in patients with addisonian pernicious anemia.

7. An "incomplete" molecule, pteroic acid, has been synthesized by omitting glutamic acid. It consists of a pteridine grouping linked to para-aminobenzoic

acid, and is biologically active for *S. fecalis* R but not for *L. casei* or for chicks or rats (table 2).

Tropical macrocytic anemia and its counterpart in monkeys. A description of "tropical macrocytic anemia" appeared in 1931 (4). The disease was found to have a blood picture similar to that in addisonian pernicious anemia but the other symptom-complexes associated with pernicious anemia were not present. The condition was observed in women patients in Bombay and was at times complicated by pregnancy. The administration of "Marmite", a concentrated extract of autolyzed yeast, 4 grams 2 to 4 times daily, was found to relieve the anemia. In a further communication (13) a similar condition was described in monkeys, the use of which animals had been undertaken to investigate the

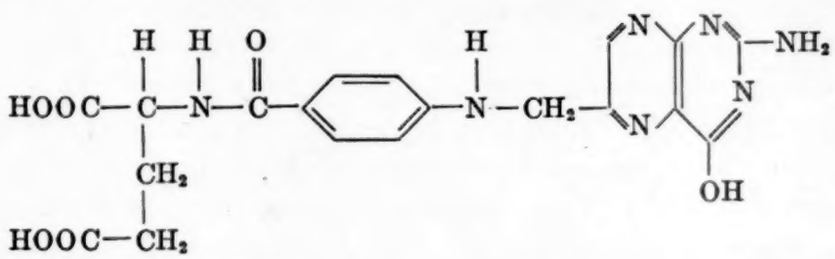

Fig. 1. Pteroylglutamic acid; N—[4—{[(2-amino-4-hydroxy-6-pteridyl)methyl]amino}-benzoyl]glutamic acid

TABLE 2

Biological activities of pteroylglutamic acid and related substances

NAME	RELATIVE ACTIVITY		ACTIVITY FOR	
	S. Fecalis R	*L. Casei*	Chick	Rat
Pteroic acid	50	0.01	−	−
Pteroylglutamic acid	100	100	+	+
Pteroyltriglutamic acid (Fermentation *L. casei* factor)	7.5	80	+	+
Pteroylheptaglutamic acid (Vitamin B_c conjugate)	0.3	0.2	+	+

nature of the missing dietary factor needed in tropical macrocytic anemia. The animals were placed on a diet similar to that consumed by women in Bombay who developed the anemia and a macrocytic anemia was produced in the monkeys which could be cured by feeding Marmite or by oral or parenteral administration of liver extracts which were active in the cure of tropical macrocytic anemia in human patients.

Studies of the bone marrow in the anemic monkeys showed a preponderance of megaloblastic cell types, and photographs of bone marrow preparations indicated a contrast between the marrow pictures respectively in monkeys with the nutritional anemia, in normal monkeys and in monkeys rendered anemic by massive hemorrhage (13). Further experiments (14) were concerned with studies of the active factor in liver; the erythrocyte and reticulocyte responses in de-

ficient monkeys were used as criteria in the assay. It was possible to differentiate the factor from the anti-pernicious-anemia factor of concentrated liver extracts; two preparations were encountered which were active for pernicious anemia in human subjects yet which produced no response in the experimental monkeys. In another article (15), the fractionation of liver extract by treatment with saturated ammonium sulfate was described; it was found that the anti-pernicious-anemia factor was precipitated, while the factor effective in monkey anemia was not precipitated. An extract prepared from yeast was subjected to similar treatment, and again the active factor for monkeys remained in solution. The factor was differentiated from thiamine, riboflavin and nicotinic acid (14).

The above observation that several types of purified liver extract which were potent in the treatment of pernicious anemia had no effect in curing the anemia in monkeys even when administered in "enormous doses" was paralleled by similar observations with human subjects with tropical macrocytic anemia. It was found that seven cases did not respond to a batch of "Anahaemin" which had been demonstrated to be effective in the treatment of pernicious anemia (16). There was no reticulocytosis, no clinical improvement, and no appreciable rise in red cell count during the 10-day period of treatment. Anahaemin has been described (17) as "a concentrated preparation of the erythrocyte maturation factor prepared by the method of Dakin and West. During precipitation it is completely precipitated with 99 per cent alcohol, taken through two ammonium sulfate fractionations, and one reineckate precipitate. Anahaemin in 2 cc. contains a fraction derived from 450 grams of fresh liver".

It has been postulated that the factor of Wills differs from both pteroylglutamic acid and the erythrocyte maturation factor (18). The evidence for the differentiation from pteroylglutamic acid was not striking; a patient failed to respond to the very small dose of 1.3 milligrams of pteroyltriglutamic acid for 10 days, following which a response was obtained to large doses of a crude liver preparation. It has been established (19, 20) that pteroylglutamic acid will cure a deficiency symptom in monkeys which appears to be similar in all respects to the condition first described by Wills and co-workers and it is also evident that pteroylglutamic acid will produce a prompt remission in nutritional macrocytic anemia and in the macrocytic anemia of pregnancy (21, 22, 23), indeed there is one report (24) describing remission of macrocytic anemia of pregnancy following treatment with pteroylglutamic acid in India under conditions which would appear to relate to the earlier observations (4). The pteroylglutamic acid content of Marmite is not known, but yeast, from which Marmite is prepared, is a good dietary source of pteroylglutamic acid and its conjugates. The preponderance of the evidence indicates that the first observation of pteroylglutamic acid deficiency was made by Wills in human subjects and that her experiments were the first to differentiate pteroylglutamic acid from the erythrocyte maturation factor of concentrated liver extracts.

Other studies of blood dyscrasias of nutritional origin in monkeys; "vitamin M". From time to time, reports of experiments with monkeys on restricted

diets have revealed the occurrence of a nutritional deficiency syndrome which was characterized by oral, gastrointestinal and hemopoietic disturbances. It was pointed out in a recent review (25) that observations were made in 1919 (26) which described diarrhea and dysentery in monkeys on diets consisting largely of autoclaved rice. Attention was also drawn (25) to the reported appearance of the syndrome in several laboratories and under various dietary regimes.

A separate series of investigations with monkeys was started in 1935 (27). In an attempt to produce riboflavin-deficiency cataract, a cooked diet of polished rice, ground wheat and purified casein was used, supplemented with cod liver oil, salt mixture and orange. A deficiency syndrome was encountered which was marked by anemia and leucopenia, ulceration of the gums, diarrhea, and susceptibility to bacillary dysentery. The disease terminated fatally within 26 to 93 days if allowed to progress. It was prevented by brewers' dried yeast, 10 grams daily or by a liver-stomach preparation, 2 grams daily (28).

It was reported (29) that riboflavin did not appreciably alter the course of the disease, termed "nutritional cytopenia", and that nicotinic acid, 10 mgm. or 50 mgm. daily, did not prevent the devlopment of the syndrome and did not postpone its fatal termination. The term "vitamin M" was proposed for the factor which prevented nutritional cytopenia in the monkey. Other features of the disease included edema (30) and susceptibility to various experimental infections (31, 32). The resistance of normal monkeys and the susceptibility of "vitamin-M-deficient" monkeys to bacterial dysentery was strikingly demonstrated (33).

An article from another laboratory (34) established the important point that leucopenia developed in monkeys which received a purified diet which was supplemented with certain vitamins, the effect of some of which had not been previously reported. The list included thiamine, riboflavin, pyridoxine, niacinamide, calcium pantothenate and ascorbic acid. The additional administration of 50 mgm. each of choline and para-aminobenzoic acid was ineffective. This report differentiated the protective factor from the known members of the vitamin B complex, with the possible exception of biotin. In addition, a new light was cast upon the possible nature of the unidentified factor; it was reported that a "folic acid concentrate" produced a white-cell response. As a result of assays of various supplements with *Streptococcus fecalis* R, doubt was later cast upon the possible identity of vitamin M with "folic acid" (25); this was, however, before studies with enzyme preparations had revealed the presence of microbiologically-inert conjugates of pteroylglutamic acid in natural foods. With the use of such enzyme preparations, prepared from rat liver, it was found that materials which were good sources of "vitamin M" were also good sources of "bound folic acid" which was liberated in the free form upon treatment with the liver preparation (35, 36) and the concept of the possible identity of the "*S. lactis* R stimulating substances together with the substances enzymatically convertable to such factors" with "vitamin M" was advanced (37). Finally it was demonstrated that pteroyltriglutamic acid, 4 or 4.5 mgm. per monkey in divided doses over a period of a few days, produced prompt and complete remis-

sion of nutritional cytopenia in monkeys, with a return of the granulocyte and total counts to normal levels, and with marked reticulocyte crises (19). Attention was drawn to the observation that the reticulocyte responses of the monkeys to pteroyltriglutamic acid were greater than the corresponding responses of pernicious anemia patients to adequate therapy (38). Monkeys on purified diets supplemented with all the known vitamin B factors except pteroylglutamic acid developed clinical and hematological signs of nutritional deficiency (20). Two of the animals were treated with pteroylglutamic acid, 0.1 to 0.35 mgm., at various intervals. Clinical and hematological remissions were observed.

Monkeys were fed a purified diet and a gradual loss of weight was observed followed by a syndrome which included anorexia, leucopenia and lowered resistance to secondary infections and which had a fatal termination. Feeding a norite eluate fraction of liver, which contained "folic acid", was found to promote growth and to alleviate leucopenia (39). More recently, studies from the same laboratory have indicated that even when a "folic acid" concentrate was supplied, further addition of whole liver was necessary for optimum growth and "blood regeneration" in monkeys (40). The activity of the liver preparation was very easily destroyed by heating (41).

Monkeys were maintained on a purified diet deficient in pteroylglutamic acid and the effect of the deficiency on experimental poliomyelitis was studied (42). An increased resistance to the disease was observed when a subacute nutritional deficiency was produced by maintaining the animals on a suboptimal level of a liver "folic acid" concentrate, but no resistance was observed in "acute" pteroylglutamic acid deficiency.

It was stated (43) that a concentrate "showing high B_{10} and B_{11} activity" for the chick was inadequate as a source of folic acid for the monkey. Presumably "B_{10} and B_{11} activity" is an expression of the presence of conjugated pteroylglutamic acid, in which case this finding may be contrasted with the parallel drawn (35, 36) between vitamin M activity and the presence of "bound folic acid" which was liberated into a microbiologically active form by enzymic digestion.

Biological effects of xanthopterin. Rats three to four weeks old when fed exclusively on goats' milk were found to develop an anemia which did not respond to supplementation with iron. The erythrocyte count fell to around 1 million per cu. mm. and the blood picture was of the macrocytic type (44). Typical reticulocyte responses were obtained with liver preparations and the condition was used as a method of testing such preparations (45). The animals showed sprue-like symptoms, so that the condition was termed "rat sprue". In another investigation of goats' milk anemia (46) fractionation of liver and human urine indicated that the active material had properties resembling those of uropterin (xanthopterin). Accordingly, a sample of uropterin was obtained from Koschara. This was injected at various levels, the effect on the erythrocyte counts was followed and graded responses were obtained over a rage of 0.5 to 1.0 microgram; 10 micrograms did not appear to cause a greater response than did 1 microgram and levels of 1 or 10 micrograms produced an elevation of 2 to 3

million in the erythrocyte count in 14 days. This experiment was the first clue to the chemical nature of pteroylglutamic acid. In contrast to the preceding report (44), the anemia was stated to be also relieved by iron and copper. Failure on the part of Rominger to confirm the results with uropterin was noted (47). However, a subsequent report (48) indicated that xanthopterin, 20 micrograms daily, produced a growth response and an increase in the white cell count in rats receiving a purified diet containing succinylsulfathiazole. Once again, difficulty in repeating these results was encountered (35, 49, 50, 51).

Xanthopterin was found to have a hemopoietic effect when 50 micrograms was injected into fingerling salmon with nutritional anemia (52). The salmon were made anemic by feeding "a high protein diet which contains yeast as a source of the vitamin B complex". The red cell counts per cu. mm. ranged from 416,000 to 916,000 for the control fish and from 659,000 to 1,305,000 for the injected fish 2 to 3 days after the injection.

A consistent but transitory response was obtained when monkeys with nutritional cytopenia were treated with xanthopterin 2.5 to 10 mgm. daily (53). The response consisted of a reticulocyte rise and a marked increase in both white and red cell counts, but the counts soon declined again, and three of the four animals died.

When fresh rat liver tissue was incubated with xanthopterin, a marked increase in the "folic acid" content of the preparation was obtained as compared with rat liver which was incubated alone or with leucopterin, adenine, guanine, xanthine, uracil or cytosine. The "folic acid" content was measured by assay with *L. casei* (50). This observation was confirmed (35), and it was noted that the liver tissue of monkeys, but not that of chicks, appeared to show a higher "folic acid" content when incubated with xanthopterin than when incubated alone. In another report, it was stated that the "folic acid" content of rat liver and muscle was influenced during digestion by various factors including neutral salts, cyanide, xanthopterin, the degree of dispersion of the tissue, the pH, the length of the digestion period and the addition of taka-diastase (54). A protective effect of xanthopterin against the destruction of "folic acid" by enzyme in rat liver was suggested (55). Another obvious variable is the possible effect of the various factors upon the action of "vitamin B_c conjugase" enzyme system (p. 79) present in rat liver, particularly evident in one experiment (54, fig. 4) in which rat liver, incubated alone at various pH values was found to yield a maximum amount of "folic acid" at pH 7.

Pteroylglutamic acid in poultry nutrition. A dietary deficiency of pteroylglutamic acid may readily be produced in young chicks on purified diets. Chicks appear to derive very little of their vitamin B-complex requirement from "intestinal synthesis", and they are, in contrast to rats, quite susceptible to a dietary lack of pteroylglutamic acid. The ease with which deficiencies were produced in chicks on purified diets led to the extensive use of this species as an experimental animal.

Starting in 1938 investigations were made to determine whether the chick needed B-complex vitamins in addition to the factors then known. The require-

ment of the chick for pyridoxine, nicotinic acid, choline and biotin was unexplored at that time. In one report (56), rice bran extract and a fullers' earth adsorbate of whey were added to a simplified basal diet. Growth was improved by a water-soluble factor which was present in alfalfa meal and which was precipitated by alcohol and adsorbed to some extent by fullers' earth, from which it was eluted by a mixture of water, acetone, and ammonia. It was found (6) that chicks grew slowly on a diet consisting principally of polished rice and washed fish meal, supplemented with thiamine and whey adsorbate, and with filtrate factor (pantothenic acid) preparations from rice bran or whey. Growth was greatly increased by yeast, or by alfalfa meal or a water extract of it. The factor, termed "Factor U", extracted from yeast, was adsorbed on fullers' earth and eluted by a mixture of pyridine, alcohol and water. Evidence for the presence of an unidentified factor in cereals, yeast and milk was advanced (57). The factor was needed for growth and hatchability, and was destroyed by prolonged dry heat. Biotin is destroyed by such treatment (58). In a further report on "Factor U" (59), pyridoxine was found to promote growth when added to the basal diet, but additional growth was produced by adding the yeast fullers' earth eluate. The presence of a growth-promoting factor in yeast distinct from thiamine, riboflavin, nicotinic acid, pyridoxine, pantothenic acid and choline was noted (60).

In another investigation (7), acid-hydrolyzed yeast, rice polishings extract, and alcohol extract of liver were used as supplements to a purified diet. Presumably the pteroylglutamic acid in the yeast was destroyed by the acid hydrolysis. A macrocytic hyperchromic anemia was developed by the chicks on this diet; it was stated that evidence indicated that it was not prevented or cured by any vitamin previously described. The antianemic factor from an undescribed source was adsorbed on fullers' earth at pH 1. The factor was given the name "vitamin B_c" (61). This investigation was important in that it reported the specific anti-anemic properties of the new vitamin and thus foreshadowed future developments.

Strong evidence that the norite eluate factor required by *L. casei* was essential for the chick was reported (62). A concentrate of the factor had a marked growth-promoting effect when added at a level of only 0.1 gram per kilo of diet. The "norite eluate factor" for *L. casei* is now presumed to be pteroylglutamic acid. It was found that disparities existed between the microbiological potency of certain concentrates and their activity for chicks. Apparently on this basis, the existence of two new vitamins, B_{10} and B_{11}, active for chicks, was postulated (11). "Vitamins B_{10} and B_{11}" were the subject of a number of publications (63, 64, 65) but in a subsequent report from the same laboratory (66) it was noted that "neither para-aminobenzoic acid nor vitamins $B_{10} + B_{11}$ gave a supplementary effect in the presence of synthetic folic acid". Presumably "vitamins B_{10} and B_{11}" represented a conjugate or conjugates of pteroylglutamic acid.

The biological activity of crystalline pteroylglutamic acid for the chick was first reported in 1943 (67). "Vitamin B_c" from liver was fed at a level of 2.5 parts per million of diet which prevented anemia and enabled good growth to

take place. The chicks which received the purified diet plus pteroylglutamic acid grew more rapidly than chicks on a "broiler ration". It was indicated (68) that 0.4 parts per million of diet was sufficient for normal hemoglobin, hematocrit, red cell count and thrombocyte values, but about a level of 4 parts was required for the production of normal leucocyte levels. A comparison of injected and orally administered dosage (69) indicated that subcutaneous injection was, if anything, slightly more effective than feeding by pipet. In further studies, it was reported that maximum growth, with submaximal hemoglobin response, was obtained by adding pteroylglutamic acid, either free or as the conjugate, at a level of 0.25 parts per million of diet (70).

The pteroylglutamic acid requirement of chicks was studied on a purified diet using growth, feathering and pigmentation as criteria (71). The vitamin was administered by injection 5 times weekly and it was found that a level calculated to supply 10 micrograms per day produced feathering and pigmentation equivalent to that observed in chicks receiving 10 per cent of brewers' yeast as a supplement to the basal diet but growth was somewhat more rapid on the yeast-supplemented diet. Growth and feathering were in proportion to the level of pteroylglutamic acid used.

Chicks on a purified diet were found to need at least one part of pteroylglutamic acid per million parts of diet for normal feather pigmentation and two parts per million to give growth equal to that obtained with a liver fraction (72). Pteroylglutamic acid, added at levels of one to five parts per million of purified diet, was found to be essential for the normal growth of feathers in chicks (73). The effect was not lessened by adding sulfamerazine or certain other "intestinal antiseptics".

In experiments with chicks on a purified diet, pteroylglutamic acid was found to produce maximum growth and hemoglobin values when added at a level of 0.5 to 1.0 mgm. per kilo of diet (74). Chicks were found to need 0.25 part of pteroylglutamic acid per million parts of purified diet for growth, feathering and hemoglobin formation (66). When sulfasuxidine was added to the diet the requirement was increased to between 0.5 and 1.0 part per million. In contrast, the pteroylglutamic acid requirement of chicks was found not to be increased by the addition of sulfasuxidine to a purified diet (75). Chicks were found to require 0.45 part of pteroylglutamic acid per million parts of diet for growth and hemoglobin formation to four weeks of age. The requirement for hemoglobin formation to six weeks of age was stated to be slightly less, 0.35 part per million and 0.55 part per million were required to produce normal feathering at six weeks of age. Attention was drawn (76) to earlier low estimates of the pteroylglutamic acid requirement of chicks (11, 63, 65, 77) which had resulted from feeding preparations of pteroylglutamic acid conjugates. It was pointed out that microbiological assays of such conjugates, even after treatment with taka-diastase, did not reveal the total pteroylglutamic acid content.

Perosis was observed in chicks on a purified diet without pteroylglutamic acid and the incidence was increased by adding 2 per cent of sulfasuxidine. The perosis was prevented by adding 0.2 part per million of pteroylglutamic acid

to the diet without sulfasuxidine and 0.3 part per million to the diet containing sulfasuxidine. The higher incidence of perosis on the sulfonamide-containing diet led to the suggestion that pteroylglutamic acid stimulated the intestinal flora to produce an unknown anti-perotic factor (78).

A relation was observed between pteroylglutamic acid deficiency in chicks and an absence of the normal response to the administration of diethylstilbestrol (79). Large doses of stilbestrol produced an only slight increase in the weights of oviducts of young chicks maintained on a purified diet which was deficient in pteroylglutamic acid, but stilbestrol produced a marked increase in the weight of the oviducts of control chicks receiving a supplement of 20 micrograms of pteroyltriglutamic acid daily. The failure of the deficient birds to respond was not due merely to inanition, for chicks with pantothenic acid deficiency and of comparable body weights showed substantial responses to stilbestrol as measured by increase in the weight of the oviduct.

Differentiation of pteroylglutamic acid from the erythrocyte maturation factor of concentrated liver extracts, previously indicated by studies with human subjects and with monkeys (14, 15, 16), was further emphasized by observations with chicks. In one investigation, an undescribed preparation of the anti-pernicious-anemia factor was found to be ineffective in the prevention of "vitamin B_c deficiency" (80) and subsequently (81) it was reported that pteroylglutamic acid was not liberated in appreciable quantities from concentrated liver extract by treatment with "conjugase" as supplied by dried chicken pancreas. The maturation factor as present in liver extract was ineffective for growth when injected into pteroylglutamic acid-deficient chicks at a level which would correspond, on an anti-pernicious-anemia basis, to about 0.4 mgm. of pteroylglutamic acid daily.

Reproduction in chickens, previously not reported with purified diets, was obtained on a diet which contained pure vitamin B complex factors including pteroylglutamic acid (82).

Young turkeys were found to develop marked nutritional deficiency on diets deficient in pteroylglutamic acid. A spastic type of cervical paralysis was observed on purified diets which were deficient in the vitamin (83). The paralysis was reversed by the administration of pteroylglutamic acid. These observations were confirmed (84) and the occurrence of a blood dyscrasia characterized by macrocytosis and elongation of the erythrocytes was described. The requirement for pteroylglutamic acid under the conditions of the experiment appeared to be at least twice as great for turkeys as for chickens.

Studies of the effects of pteroyltriglutamic acid in chicks. This compound was found to be "active in the nutrition of the chick" (85).

It was reported that pteroyltriglutamic acid, when added at the rate of 0.5 mgm. per kilo of purified diet, was only partially effective in promoting growth and preventing anemia (86). However, when the lactone of either 5-pyridoxic acid (2-methyl-3-hydroxy-4-hydroxymethyl-5-carboxy pyridine) or 4-pyridoxic acid (2-methyl-3-hydroxy-4-carboxy-5-hydroxymethyl pyridine) was added at a level of 0.5 mgm. per kilo to the diet together with pteroyltriglutamic acid,

termed *L. casei* factor, at the same level, a marked gain in weight was produced and anemia was completely prevented. The names alpha and beta "pyracin" were proposed for the two pyridoxic acids.

Hens on a commercial diet were made anemic by bleeding, and the rate of regeneration of hemoglobin was followed (87). The injection of either 4-pyridoxic acid or pteroyltriglutamic acid, 50 micrograms daily, hastened the rate of the hemoglobin level, and when these supplements were administered together, the rate was still further increased.

In another communication, the incubation of pteroyltriglutamic acid with fresh chick liver was found to result in an increase in "folic acid content" as measured with *S. fecalis* R. There was a further increase when 4- or 5-pyridoxic acid was added but a diminution when pyridoxic acid lactone was added rather than the acid (88). Pteroylglutamic acid was found to be effective against anemia in chicks without the addition of pyridoxic acid lactone to the diet (89, 76). Evidence was obtained with chicks (90) which tended to confirm the report of the supplementary effect of 4-pyridoxic acid on the utilization of pteroyltriglutamic acid. The experimental data were meager due to the small amount of pteroyltriglutamic acid which was available.

Other workers reported that pteroyltriglutamic acid without added pyridoxic acid was an effective as pteroylglutamic acid, on a molar basis, in promoting growth and preventing anemia in chicks (74) (91). As compared with pteroylglutamic acid, pteroyltriglutamic acid appeared to be completely utilized with or without the addition of 4-pyridoxic acid by chicks on a diet similar to that described by the Cornell group (91). It has also been found that pteroylheptaglutamic acid was utilized as efficiently as pteroylglutamic acid, without the addition of pyridoxic acid, by chicks on a purified diet (92).

Rôle of pteroylglutamic acid in microbiological nutrition. A summary of the pteroylglutamic acid requirements of microorganisms is given in table 3.

A large number of lactic acid bacteria have been shown to need pteroylglutamic acid for growth. In an investigation of the nutritive requirements of lactic acid bacteria (8) it was shown that an unidentified growth factor existed which later proved to be pteroylglutamic acid. Later it was shown at the same laboratory that this factor was required by other lactic- and propionic-acid-forming bacteria *Streptococcus fecalis* R, *Lactobacillus delbruckii*, and *Propionibacterium pentosaceum* (93).

Clostridium tetani was found to require a growth factor which could be replaced by concentrates of pteroylglutamic acid made from liver (94) or by a "folic acid" preparation obtained from spinach (95). Three "folic acid" preparations of widely varying potencies as measured with *S. fecalis* R were found to have corresponding activities for *Cl. tetani* thus indicating that pteroylglutamic acid was the effective agent.

The growth requirements of a large number of enterococci was investigated (96) and it was found that of 43 organisms investigated 9 required pteroylglutamic acid. It was also found by the same investigators that of 21 strains of *Streptococcus lactis* none required pteroylglutamic acid (97). The requirements

of a large number of lactic acid bacteria for pteroylglutamic acid and the "S. L. R factor" were investigated (99, 104) and are described in detail elsewhere in this review.

The nutrition of *Tetrahymena geleii* has been thoroughly investigated and this organism was shown to require pteroylglutamic acid (106, 110). The amount required for half maximum growth is 0.00065 microgram per ml. of culture medium which is about three times the amount required for half maximum growth by *S. fecalis* R. (10). The "S. L. R factor" which is active for *S. fecalis* R but relatively inactive for *L. casei* was found to be about 0.2 per cent as active as

TABLE 3

Activity of pteroylglutamic acid and related compounds for microorganisms

MICROORGANISMS	PTEROYLGLUTAMIC ACID	PTEROYL-TRI-GLUTAMIC ACID	PTEROYL-HEPTA-GLUTAMIC ACID	PTERGIC ACID	S. L. R. FACTOR	THYMINE
Clostridium tetani....	+(94,95,100)					
Lactobacillus casei...	+(67)	+(85)	−(70)	−(101)	−(102)	+(103)
L. helveticus.....	+(93)					
L. delbruckii.....	+(93)					
L. delbruckii LD5....	+(104)					
L. bulgaricus D5.....	+(104)					+(105)
Propionibacterium pentosaceum.....	+(93)					+(105
Streptococcus durans 98A.....	+(104)				+(104)	+(105)
S. fecalis 732.....	+(104)				+(104)	+(105)
S. fecalis F-24.....	+(104)				+(104)	+(105)
S. fecalis R.....	+(104)	±(85)	−(70)	+(101)	+(104)	+(105)
S. fecalis S-108A.....	+(104)				−(104)	+(105)
S. zymogenes 5Cl.....	+(104)				+(104)	+(105)
Tetrahymena geleii...	+(106)		+(108)	−(108)	−(107)*	
Yeast "Old process".....	+(109)					

* Approximately 0.2 per cent as active as pteroylglutamic acid.

pteroylglutamic acid for *T. geleii* (110). Later it was reported by the same workers (108) that pteroylheptaglutamic acid was active. This contrasts *T. geleii* with *S. fecalis* and *L. casei* which are unable to utilize this conjugate. Thus the ability of *T. geleii* to utilize the conjugate is similar to that of animals, and parallels the similarity of *T. geleii* and the higher animals in amino acid requirements (111).

It seems likely that those organisms which do not require pteroylglutamic acid are able to synthesize it. *Bacillus lactis acidi*, *Lactobacillus arabinosus*, *Lactobacillus pentosus*, *Bacillus brassicae*, *Leuconostoc mesenteroides* and *Lactobacillus gayonii* are not stimulated by pteroylglutamic acid but were able to synthesize significant amounts of the factor (93).

The synthesis of pteroylglutamic acid by five microorganisms was reported (112). The assays were made after papain and takadiastase digestion of the cells and using *S. fecalis* R as the assay organism. This enzyme treatment would presumably not hydrolyze the conjugates present so the results must be interpreted to measure primarily free pteroylglutamic acid and any S. L. R factor which may be present. The organisms were grown on a purified media containing no pteroylglutamic acid and the cells and media assayed separately. The results were as follows:

ORGANISM	MICROGRAMS PTEROYLGLUTAMIC ACID FOUND PER GRAM DRY CELLS*	
	Cells	Medium
Aerobacter aerogenes (aerobic)	5	31
Aerobacter aerogenes (anaerobic)	2	7
Serratia marcesans	6	28
Pseudomonas fluorescens	3	23
Proteus vulgaris	7	7
Clostridium butylicum	1	6

* Calculated on the basis of pteroylglutamic acid having a "potency" of 137,000.

The growth-promoting activity of pteroylglutamic acid for *S. fecalis* R was reported to be reversed by the addition of a synthetic product, "methylfolic acid", which was prepared by reacting 2,4,5-triamino-6-hydroxy pyrimidine and *p*-aminobenzoyl-d(−)-glutamic acid with 2,3-dibromobutyraldehyde (113).

A relationship was established between pteroylglutamic acid synthesis and sulfanilamide in *E. coli* (114). A sulfonamide resistant and a sensitive strain of *E. coli* were grown in concentrations of sulfanilamide which permitted some growth. Both strains produced less pteroylglutamic acid when grown in the presence of sulfanilamide than in the absence of it. Biotin synthesis on the other hand was not greatly affected by the presence of the drug which demonstrated that the sulfanilamide did not exert a general depressing action on synthesis of all the vitamins.

While pteroyltriglutamic acid and pteroylglutamic acid were approximately equally active for *L. casei*, the shapes of their respective response curves were different (115). There was a lag in the response to low concentrations of pteroyltriglutamic acid which resulted in a sigmoid shaped curve. The lower part of the response curve with pteroylglutamic acid approached a straight line. A sample of "folic acid, potency 3100" obtained from spinach gave the same shape response curve as that of pteroylglutamic acid, and on this basis it was suggested that the active constituent in the "folic acid" preparation was pteroylglutamic acid. Comparative assays with *L. casei* and *S. fecalis* R showed the "folic acid" preparation to have the same relative potency as pteroylglutamic acid for these two organisms. By using a folic acid preparation with potency standardized in terms of a liver fraction the "potency" of pteroylglutamic acid was estimated to be approximately 137,000.

Para-aminobenzoic acid and its relation to pteroylglutamic acid in bacterial nutrition. There is evidence that p-aminobenzoic acid functions as a precursor of pteroylglutamic acid in the nutrition of certain bacteria. The bacteriostatic effect of the sulfonamides is due in the case of some microorganisms to inhibition of the enzyme system which synthesizes pteroylglutamic acid from p-aminobenzoic acid.

A possible indication of the relation between p-aminobenzoic acid and pteroylglutamic acid appeared before the chemical nature of pteroylglutamic acid was revealed. It was reported (116) that a yellow pigment was formed in cultures of a certain strain of *Mycobacterium tuberculosis* when it was grown in media containing high concentrations of p-aminobenzoic acid, and it was suggested that the pigment might be related to the vitamin B complex. It was also suggested that the enzyme responsible might be a specific oxidase (117). The pigment was reported to contain oxidized aromatic nitrogen groups as indicated by an increase of aromatic amino nitrogen after treatment with zinc. The pigment gave negative results for the presence of "folic acid" when tested with *S. fecalis* R. This negative result presumably would not exclude the possible presence of certain conjugates of pteroylglutamic acid which are known to be of quite low activity for *S. fecalis* R. Pteroylheptaglutamic acid (Table 1) is an example of such a conjugate. The formation of the pigment could be lessened or inhibited by sulfanilamide (118). It was noted that a similar culture of this strain of *M. tuberculosis* contained a fraction which promoted growth in chicks. This effect was attributed to considerable amounts of "B_{10} and B_{11}" rather than to pteroylglutamic acid (64); the relation between pteroylglutamic acid and "B_{10} and B_{11}" (66) has not then been evaluated. It thus appeared possible that an effect of p-aminobenzoic acid upon this strain of *M. tuberculosis* was to increase the production of conjugated pteroylglutamic acid. The nature of the yellow pigment (116) has not yet been described.

A mixed culture of organisms was obtained from the duodenum of chicks. The growth rate of the culture was increased by p-aminobenzoic acid to the medium, and simultaneously a three-fold increase in folic acid production as measured by assay with *S. fecalis* R was observed (119).

It was noted that the growth of *S. fecalis* R and *L. casei* in the presence of pteroylglutamic acid, pteroyltriglutamic acid or pteroic acid was affected very little by sulfonamides (120). Growth of these organisms did not take place if p-aminobenzoic acid or p-aminobenzoyl glutamate was substituted for pteroylglutamic acid, pteroyltriglutamic acid or pteroic acid. This indicated that these organisms cannot form pteroic acid or pteroylglutamic acid from p-aminobenzoic acid or p-aminobenzoyl glutamate and that they need preformed pteroic acid or pteroylglutamic acid. Hence there is no opportunity for a sulfonamide to compete with p-aminobenzoic acid in the nutrition of these organisms. With another organism, *S. fecalis* Ralston, which responded to p-aminobenzoic acid, p-aminobenzoyl glutamate, pteroylglutamic acid, pteroyltriglutamic acid or thymine, competitive inhibition between sulfadiazine and p-aminobenzoic acid or p-aminobenzoyl glutamate was observed. In the presence of pteroylglutamic acid,

pteroyltriglutamic acid or thymine, the organism became highly resistant to sulfadiazine, indicating that the inhibitory action of sulfadiazine was due to interference with the formation of pteroylglutamic acid from p-aminobenzoic acid and that when preformed pteroylglutamic acid was supplied, the organism did not "need" to carry out this synthesis and hence sulfadiazine was noninhibitory. The observations with thymine recall the previous observation that for certain bacteria this substance in the absence of pteroylglutamic acid is a growth promoting factor, and the suggestion that the function of pteroylglutamic for these bacteria is to catalyze the formation of thymine (p. 66). A third type of organism, represented by *E. coli*, did not require preformed pteroylglutamic acid, and the bacteriostatic effect of sulfadiazine on this type was reversed by p-aminobenzoic acid but not by pteroylglutamic acid, which observation might be presumed to indicate that these organisms could not utilize preformed pteroylglutamic acid.

Thymine and pteroylglutamic acid. A relation between the pyrimidine base, thymine (5-methyl uracil) and "folic acid" was observed in studies with *S. fecalis* R (121). This lactic acid organism was enabled to grow by the addition of both thymine and a purine base. Thymine could not be replaced by uracil, but the purine requirements were less specific, adenine and guanine being almost interchangeable.

Thymine was also found to have a growth-promoting effect on *L. casei* (103). A combination of both thymine and a purine base was necessary. Approximately 0.5 microgram of thymine and 5.0 micrograms of guanine per ml. of media were required to give the maximum response. The maximum growth obtained with thymine and purines was only half that obtained with concentrates of *L. casei* factor (pteroylglutamic acid). Thymine could not be replaced by uracil or cytosine while the purine requirements were met by guanine, xanthine, adenine or hypoxanthine.

A report on the requirements of *S. fecalis* R showed that thymine could not be replaced by 4-carboxyuracil, 4-carboxythymine, or 5-carboxyuracil (109). Nucleic acid was found to be inactive as a source of thymine for *S. fecalis* (122), indicating the inability of the organism to utilize thymine in the form of a nucleic acid. While no data has appeared on the activity of thymine nucleotide, the nucleoside thymidine has been shown to be as active as thymine on a molal basis (105). In this report the inactivity of a number of pyrimidines was also described.

The interfering effect of thymine on the assay of pteroylglutamic acid with *L. casei* received comment (123).

A possible role of thymine in serving as a substitute for "folic acid" was formulated on the basis of experiments with these two compounds in the nutrition of *S. fecalis* R and *L. casei* (105). It was found that while pteroylglutamic acid alone would give a response, addition of a purine was needed to give maximum response. In the presence of a purine, approximately 5,000 times as much thymine as pteroylglutamic acid was required. With *S. fecalis* R, thymine permitted the same maximum growth as was obtained with pteroylglutamic acid,

but with *L. casei* thymine *plus* a purine gave only half the maximum response produced by pteroylglutamic acid.

The possibility of formation of pteroylglutamic acid by *S. fecalis* R cells during growth on thymine was tested by autoclaving the cells with dilute hydrochloric acid and assaying with *L. casei*. A "plateauing" of the *L. casei* assay response at approximately half-maximum growth was characteristic of the response obtained with thymine and suggested that the material in the *S. fecalis* R cells giving the response was thymine and not pteroylglutamic acid. These observations led to the suggestion that pteroylglutamic acid functioned directly or indirectly as a coenzyme in the synthesis of thymine or related compounds by *S. fecalis* R. Three to four micrograms of thymine were taken up per mg. of *S. fecalis* R cells during growth, an amount which may be compared with the value of about 2.0 micrograms of thymine present per milligram of dried tubercle bacillus (124).

Studies of the effect of a large number of pyrimidines on the growth of *L. casei* were reported (125). Derivatives of thymine in which one or both of the oxygen atoms were replaced by an imino or thio group in many cases promoted the growth of *L. casei*. The replacement of oxygen by the imino group appeared to lower the activity to about 0.1 that of the corresponding oxy compound. Other relationships of the effect of chemical structure upon biological activity were discussed. It was found that isobarbituric acid and 5-amino uracil reversibly antagonized the growth promoting effect of thymine or pteroylglutamic acid. It was also observed that bromouracil could inhibit completely the growth of *L. casei* with thymine as the nutrient but had no effect or produced slight stimulation when pteroylglutamic acid was used as a nutrient. This observation did not support the hypothesis (105) that the function of pteroylglutamic acid in promoting the growth of lactic acid bacteria was to enable the organisms to synthesize thymine. It was further observed (125) that nitrouracil at certain concentrations could antagonize the growth-promoting action of pteroylglutamic acid but had no effect on the growth-promoting action of thymine. Thymine in large doses has been reported to cause hemopoietic remission in pernicious anemia and sprue (pp. 84, 90); the substance appears to be ineffective in preventing pteroylglutamic acid deficiency in rats and chicks (126, 127).

The "Streptococcus lactis R factor". A factor was described which was highly active for *S. fecalis* R, but relatively inactive for *L. casei* (102). One microgram of this "S. L. R factor" had an activity for *S. fecalis* R equal to 1.25 micrograms of "folic acid (potency 137,000)" but for *L. casei* it had an activity equal to less than 0.00001 microgram of the same preparation of folic acid. Pteroylglutamic acid has been shown to have the same potency as a "folic acid preparation of potency 137,000" (115).

A survey was made of the "S. L. R factor" and pteroylglutamic acid requirements of a number of lactic acid bacteria (104). The results which appear in table 4 revealed that those organisms which are able to utilize the "S. L. R factor" can also use pteroylglutamic acid. A certain number, mostly Lactobacilli, were able to utilize pteroylglutamic acid only.

These results also demonstrated that different strains of the same organism have widely differing requirements. Thus one strain of *S. fecalis* can use either the "S. L. R factor" or pteroylglutamic acid, another requires pteroylglutamic acid while a third requires neither. Synthesis of pteroylglutamic acid was stated to be established for three of these strains.

It was also reported that certain enterococci were able to convert the "S. L. R factor" into a form which is active for *L. casei*. It was found that 0.06 microgram of this factor per 10 ml. of media did not permit growth of *L. casei* but that 1 ml. of a fluid culture of "*S. fecalis*" grown with 0.006 μg per ml. of the factor would permit rapid growth. This demonstrated that *S. fecalis* R had converted the "S. L. R factor" into a form active for *L. casei*, which was presumed (104) to be pteroylglutamic acid. In the same report it was stated that incubation of the "S. L. R factor" with rat liver suspensions did not give rise to "folic acid".

TABLE 4

Streptococcus lactis R factor and pteroylglutamic acid requirement of lactic acid bacteria

Organisms requiring:

"S. L. R. FACTOR" OR PTEROYLGLUTAMIC ACID (INTERCHANGEABLE)	PTEROYLGLUTAMIC ACID	NEITHER
Streptococcus lactis R	*Lactobacillus casei*	*Lactobacillus arabinosus* 17–5
Streptococcus fecalis 732	*Lactobacillus delbruckii* LD5	*Leuconostoc mesenteroides* 6205
Streptococcus fecalis F24	*Lactobacillus bulgaricus* O5	*Streptococcus lactis* 374, 4487, 8039, 7963, 4386, L103, L104, L206
Streptococcus zymogenes 5Cl	*Streptococcus casei* 19	*Streptococcus fecalis* 10Cl
Streptococcus durans 98A	*Streptococcus fecalis* S108A	*Streptococcus zymogenes* 6054

This constituted evidence for the belief that the "S. L. R factor" is not a conjugate of pteroylglutamic acid since liver enzymes are capable of cleaving conjugates.

In a later paper from the same laboratory a detailed study of the conversion of the "S. L. R factor" to pteroylglutamic acid by various enterococci was reported (99). The ability of these organisms to effect this conversion varied widely. Resting cell suspensions of these organisms in phosphate buffer were used. A 10 ml. suspension of *S. lactis* R or *S. zymogenes* converted 5 micrograms of S. L. R factor to about 1.0 microgram of pteroylglutamic acid[1] in three hours while *S. fecalis* 232 and *S. durans* 98A formed only 0.18 microgram. The addition of carbohydrates, e.g. *d*-ribose, glucose and fructose increased the formation of pteroylglutamic acid 5- to 15-fold. Other carbohydrates tested permitted growth but did not accelerate the formation of pteroylglutamic acid. Those

[1] The data have been recalculated on the basis of pteroylglutamic acid assuming that pteroylglutamic acid has the same activity as folic acid of potency 137,000.

sugars which could act as hydrogen acceptors as evidenced by reduction of methylene blue during the incubation with resting cells, were the ones capable of increasing the rate of conversion. Sucrose was unable to stimulate this conversion by resting cells which had been grown on glucose. However, if the organism were initially grown on sucrose, then sucrose was as efficient as glucose in stimulating the conversion of the S. L. R factor, and methylene blue was reduced by the product obtained when sucrose was incubated for three hours with resting cells.

In the assay of pteroylglutamic acid formed by conversion from the "S. L. R factor" the entire cell suspensions were added directly to the assay medium without any preliminary treatment. Practically all this activity was found to be within the cells. When these cells were autoclaved with water only 12 per cent of the activity was recovered and only 28 per cent when autoclaved with phosphate buffer. However, when the cells were autoclaved in the assay medium, in 0.5 per cent sodium thioglycollate solution or in 5 per cent ascorbic acid and then assayed, complete recovery was obtained. This suggests that reducing substances exerted a protective influence during extraction from the cell. However, the residual pteroylglutamic acid in the liquid phase after autoclaving was resistant to further autoclaving. These results emphasize the difficulties that can be encountered in assaying microbial cells and the care needed to assure complete extraction.

The "S. L. R factor" ("rhizopterin") was characterized as 4(((2-amino-4-hydroxy-6-pteridyl)methyl)formamido) benzoic acid; a compound of pteroic and formic acids (306). It is inactive for hemopoiesis in sulfonamide-treated rats and pteroylglutamic acid-deficient chicks (128). Pteroic acid is similarly inactive for the chick (1). There appears to be a small difference in the relative potencies of the S. L. R factor and pteroic acid for *S. fecalis* R and *L. casei*. The activities of the "S. L. R factor" when directly compared with pteroylglutamic acid have not been reported and the only evidence available is based on recalculation of the data obtained with "folic acid" with a potency of 40,000 obtained from spinach. Using this data, the "S. L. R factor" is 125 per cent as active as pteroylglutamic acid for *S. fecalis* R and only 0.001 per cent as active for *L. casei*. Pteroic acid is 50 to 100 per cent as active as pteroylglutamic acid for *S. fecalis* R depending on the time of incubation (127) and 0.01 per cent as active by *L. casei* assay.

Pteroylglutamic acid in the nutrition of rats. On purified diets which do not contain pteroylglutamic acid, the dietary need of the rat for this factor appears to be satisfied by the production of pteroylglutamic acid by the intestinal bacteria. The addition of any of several sulfonamides to the diet depresses the growth of the intestinal bacteria and results in the appearance of certain deficiency syndromes, one of which is characterized by agranulocytosis, leukopenia, anemia and slow growth. Administration of pteroylglutamic acid or of its conjugates cures this syndrome. Some difficulties of interpretation were encountered until the fact was appreciated that certain conjugates are microbiologically inactive. A relationship may exist between the utilization of pantothenic acid and pteroylglutamic acid in rats on such diets. Lactation leucopenia

due to pteroylglutamic acid deficiency has been observed in rats on purified diets.

It was found that rats would grow and reproduce on a purified diet (129). Shortly thereafter, it was shown that when sulfaguanidine was added to a similar diet, the growth of young rats was greatly reduced and could be restored by the addition of liver extract (130). A concentrate of "folic acid" was fed at the rate of 5 milligrams daily to rats on a purified diet containing 1 per cent of sulfasuxidine (131). Definite growth responses were observed when biotin was also fed. Similar responses were obtained with a more concentrated "folic acid" preparation. In addition to promoting growth (132), "folic acid" concentrates were found to restore the color of the hair in black rats which had become grey on diets containing sulfaguanidine. The depressed growth under such conditions was found to be counteracted by feeding yeast, yeast extract or rat feces (133). Specific signs were described in rats on such diets (134), including agranulocytosis, leucopenia, anemia and hypocellularity of the bone marrow. The changes were prevented or reversed by a liver fraction which was precipitated from aqueous solution by addition of 80 per cent ethanol. A crude norite eluate containing "folic acid" was found to improve the leukocyte picture (49). These findings were confirmed with a "folic acid concentrate" (135) which was found to exert an effect similar to that of liver extract in promoting growth and preventing leucopenia in rats on diets containing sulfasuxidine. In another investigation (136) signs of pantothenic acid deficiency were observed in rats on purified diets with sulfasuxidine and the pantothenic acid content of the liver tissue of the rats was lowered, although the diet contained 40 parts of pantothenic acid per million. When biotin and a "folic acid" concentrate were added to the diet, growth was improved and the pantothenic acid content of the liver was increased. These additions were also reported to lower the prothrombin time (137). The signs of pantothenic acid deficiency, including porphyrin-caked whiskers, were found to be removed by supplementation with biotin and a "folic acid" concentrate (138), but not by additional amounts of pantothenic acid.

It was shown (50) that pteroylglutamic acid or pteroyltriglutamic acid, 20 micrograms per day for 4 days, were effective in markedly increasing the granulocytes and the total white cell count, and in relieving the anemia occurring in rats on purified diets containing sulfaguanidine or sulfasuxidine.

The anemia induced by bleeding was studied in rats which were fed a purified diet containing sulfasuxidine (139). The administration of pteroyltriglutamic acid was found to have a preventive and corrective effect on the anemia as judged by measurement of hemoglobin concentration, hematocrit readings and white counts. These observations may indicate a need for further studies of the possible role of pteroylglutamic acid in the regeneration of hemoglobin following bleeding.

Attention was drawn to the unexpectedly high activity of milk in preventing "folic acid" deficiency in rats on a purified diet containing sulfasuxidine (140). The "folic acid" content of the milk was found to be quite low as determined by assay with *L. casei* and *S. fecalis* R. When the milk was treated with "conjugase" the "folic acid" content was found to be increased more than 20-fold

(141) which indicated that the preceding observation could be explained on the basis of utilization of conjugated pteroylglutamic acid by the rat.

Storage of pteroylglutamic acid in the liver of rats was reduced by adding sulfasuxidine to a purified diet and the folic acid content of the liver was increased when a liver fraction was added to the diet (142). A series of investigations (143, 144, 145, 146) emphasized the relationships between pteroylglutamic acid and other dietary ingredients. It was shown that granulocytopenia due to pteroylglutamic acid deficiency was obtained in a small percentage of rats which were fed purified diets without sulfonamides. The dyscrasia was also produced by feeding purified diets which were low in pantothenic acid; under these conditions anemia, leucopenia, granulocytopenia, and bone marrow hypoplasia were prevented by adding pantothenic acid. It was considered that the primary deficiency producing granulocytopenia under these experimental conditions was that of pteroylglutamic acid. The administration of pantothenic acid appeared to prevent the development of pteroylglutamic acid deficiency. Riboflavin-deficient rats were found to become anemic, granulocytopenic or both. The granulocytopenia was corrected by pteroylglutamic acid and the anemia, somewhat less consistently, by riboflavin. Severe granulocytopenia and anemia were produced in rats fed protein-free diets. The condition was prevented by casein but not by pteroylglutamic acid or pteroyltriglutamic acid. Casein did not correct the granulocytopenia but when casein and pteroylglutamic acid were both added the granulocytopenia was cured.

Anemia, leucopenia, and hemorrhage and necrosis of the adrenals were observed in rats on a purified diet containing thiourea. The leucopenia and granulocytopenia were not prevented by thyroid powder or thyroxin but were corrected by treatment with pteroylglutamic acid. Pteroyltriglutamic acid, 100 micrograms daily, was effective, but treatment for 4 days with 24 micrograms did not consistently correct the dyscrasia (147). These results may be contrasted with the reported ineffectiveness of pteroylglutamic acid in preventing the onset of agranulocytosis in two patients receiving thiouracil (148).

A description of the pathology of the bone marrow of rats as affected by pteroylglutamic acid deficiency was given (149). In the deficiency the marrow was hypocellular and showed general depletion especially of the myeloid series, although the erythroid series was usually also depleted even in the absence of anemia. Administration of pteroylglutamic acid resulted in a characteristic proliferation and regeneration and the marrow returned to normal after temporary over-compensation.

Hypochromic anemia was induced by promin and promizole in young rats on a purified diet. The administration of pteroylglutamic acid, 18 micrograms daily, exerted an anti-anemic effect (150).

It was reported that concentrates of pteroylglutamic acid improved the performance of rats during lactation (151). "Lactation leucopenia" in rats on purified diets was found to be partially prevented by addition of brewers' yeast or liver extract (152) and subsequently pteroylglutamic acid was found to have

a similar effect (153) although the effect was not as complete as that of a liver fraction.

Intestinal synthesis of "folic acid" in rats on purified diets, was studied by examination of the cecal contents (154). Dextrin produced the largest amount of synthesis. A marked decrease in synthesis was caused by adding 2 per cent sulfathalidine to the diet. Increases were produced by adding niacin, lactose or milk powder to the basal diet; all these increases were prevented by adding sulfathalidine.

In experiments with rats a liver fraction was encountered which had a marked effect in relieving pteroylglutamic acid deficiency in rats which received a purified diet with sulfonamides. Its effectiveness appeared to be in excess of its pteroylglutamic acid content as measured by microbiological assay, even after treatment with acid, alkali, or a "conjugase" preparation had been used to liberate pteroylglutamic acid (155).

Isolation of pteroylglutamic acid and related compounds. Methods for the concentration of the "norite eluate factor" (pteroylglutamic acid) were described by the Wisconsin workers. In their first report (8) this factor was found to be relatively stable to acid and alkali; it could be adsorbed on activated charcoal and Lloyd's reagent; it was precipitated by phosphotungstic acid and by basic lead acetate and could be extracted from an acid solution by butanol. A later publication from the same laboratory (93) described additional methods of purification by adsorption on Superfiltrol. The factor was also concentrated from liver by adsorption and elution with norite and precipitation of the manganese salt with manganese chloride and ethanol (103).

The isolation of a highly active preparation of "folic acid" from spinach was reported by the Texas group (9) and was later described in more detail by the same group of workers (109, 156, 157). These workers employed repeated adsorption and elution from activated charcoal, precipitation with silver and lead, adsorption on Lloyd's reagent, chromatographic adsorption on alumina and precipitation of the free acid from cold acidic solutions. Since "folic acid" from spinach has never been crystallized it is not possible to establish its identity with pteroylglutamic acid. The activity of the material described in the first publication (9) had a "potency" of 40,000 as compared with a sample of a liver fraction B which was arbitrarily given a potency of 1. In a subsequent paper (156) a preparation with a "potency" of 137,000 was reported. While the activity of this preparation has not been given, a report (115) stated that synthetic pteroylglutamic acid gave a "potency" of approximately 137,000 when compared with a sample of "folic acid" of known potency obtained from spinach. Another communication (158) presented data showing that pteroylglutamic acid would have a "potency" of approximately 160,000. This data would indicate that the final product which was obtained from spinach and which had a potency of 137,000 was almost pure.

Two reports of the isolation of pure pteroylglutamic acid from liver have been made (10, 67). This compound was also isolated from a yeast concentrate which

had been enzymatically hydrolyzed (92). This yeast concentrate contained "B_c conjugate" which was active in preventing anemia in chicks but which possessed little microbiological activity until after it had been hydrolyzed enzymatically. "Vitamin B_c" isolated from a hydrolyzed yeast concentrate was identical with pteroylglutamic acid obtained from liver.

Two different methods have been described for the isolation of pteroylglutamic acid from liver. One of these starts with whole liver in which the conjugates have been converted into free pteroylglutamic acid by autolysis (67, 159). The isolation process involved essentially the following steps: extraction with boiling water, adsorption on and elution from Amberlite 4R, adsorption and elution using activated charcoal, extraction of an aqueous solution of the free acid with butanol at pH 3 to 4, formation of a barium salt, extraction of barium salts with hot water, formation of a zinc salt and crystallization of the free acid from water. The other method (10, 160) employed as the starting material an 80 per cent-alcohol-insoluble fraction of an aqueous extract of liver. The fraction contained approximately 20 micrograms of pteroylglutamic acid per gram. The pure substance was obtained by the following steps: adsorption and elution with norite, adsorption and elution with Superfiltrol, formation of a barium salt with barium chloride and methanol, esterification of the barium salt with 0.2N HCl methanol, extraction of the methyl ester from aqueous solution with butanol, chromatographic adsorption of the ester on Superfiltrol, and fractional precipitation of the ester from water and from methanol. The free acid obtained by hydrolysis of the ester was crystallized from hot aqueous solutions.

Properties of pteroylglutamic acid. Pteroylglutamic acid crystallizes as yellow spear shaped platelets. Its solubility (160) as the free acid is 10 micrograms per ml. at 0°C and more than 500 micrograms at 100°C. The sodium salt is much more soluble, having a solubility greater than 15 mgm. per ml. at 0°C. Pteroylglutamic acid has characteristic ultraviolet absorption spectra. In 0.1N NaOH it exhibits maxima at 257, 282, and 365 mμ and corresponding E (1%) 1 cm values of 585, 570, 206. Another report (161) described the absorption spectrum at pH 11. This possessed maxima at the same points but with absorption coefficients which were slightly higher; 603, 600 and 213 at wavelengths of 256, 282, 365 mμ.

The isolation of pteroyltriglutamic acid (85, 162) was accomplished by a method different from that used for the compound obtained from liver. The starting material was a cell-free filtrate obtained from aerobic fermentation of a diphtheroid-type organism. The method involved adsorption on charcoal, formation of a barium salt, esterification and extraction of the methyl ester from an aqueous solution with butanol and fractional precipitation of the ester from hot methanol. The methyl ester was dissolved in hot methanol and precipitated by cooling. In the presence of 0.05N sodium chloride the ester precipitated in a flocculent form while in the absence of electrolytes a gel was formed. After several such precipitations from methanol the ester was obtained in a microcrystalline form. The free acid was obtained in the pure state by converting the ester to the barium salt, removing extraneous pigments with Florosil and

precipitating the free acid at pH 2.8. The free acid could be crystallized from water containing electrolytes such as sodium or calcium chloride. In the absence of electrolytes gels were obtained. The pure ester was obtained by esterification of the free acid and crystallization of the ester from 0.05N sodium chloride in methanol.

While pteroylglutamic acid and its ester can be precipitated from water and methanol respectively in the absence of electrolytes, pteroyltriglutamic acid and its ester require the presence of electrolytes for their precipitation. The solubilities of pteroyltriglutamic acid in the presence of $CaCl_2$ at pH 2.8 are 3.0 mgm. per ml., at 80°C and 0.10 mgm. per ml. at 5°C.

A conjugate was isolated from yeast (70) and was later reported to contain seven glutamic acid residues (3). This compound was crystallized from 5 per cent sodium chloride. The use of electrolytes in crystallization of this compound was similar to that reported for the triglutamic acid derivative (162). The heptaglutamic acid conjugate possessed an activity for chicks which was proportional to its content of pteroylglutamic acid (70). Its microbiological activity was 0.3 to 0.6 per cent of that of pteroylglutamic acid by assay with *L. casei* and 0.2 per cent by assay with *S. fecalis* R (see table 2). The conjugate is as active on a molal basis as pteroylglutamic acid in promoting the growth of *Tetrahymena geleii* (108).

Degradation. The relationship between pteroyltriglutamic acid and pteroylglutamic acid was shown by anaerobic alkaline hydrolysis (163). The triglutamic acid derivative is active for *L. casei* but only slightly active for *S. fecalis* R (table 2). It was found that while aerobic alkaline hydrolysis produced rapid biological inactivation for both organisms, anaerobic hydrolysis produced only a slight decrease in the activity for *L. casei* and greatly increased the activity for *S. fecalis* R. The ratio of the activity for these two organisms approached that of pteroylglutamic acid isolated from liver. Two mols of alpha-amino acid nitrogen were liberated during anaerobic alkaline hydrolysis and the active compound which was formed was approximately half as active as pteroylglutamic acid by both *L. casei* and *S. fecalis* R assay. This compound was later identified as racemic pteroylglutamic acid.

Aerobic alkaline hydrolysis (163) of pteroyltriglutamic acid or racemic pteroylglutamic acid resulted in the formation of a fluorescent pigment and a diazotizable aromatic amine which could be estimated by the method of Bratton and Marshall (164). In the absence of oxygen no diazotizable amine or fluorescent pigment were produced by alkaline hydrolysis. The fluorescent pigment proved to be a dibasic acid having pKa values of 3.9 and 7.7. Elementary analysis suggested the empirical formula $C_7H_5N_5O_3$. Decarboxylation of the fluorescent dibasic pigment at 300° resulted in the liberation of approximately 1 mol of CO_2 and the formation of a fluorescent monobasic acid with a pKa of 8.0. Oxidation of the original dibasic acid with chlorine water, followed by hydrolysis with 0.1N HCl at 140°C yielded a compound which gave a positive test for guanidine. The formation of guanidine under such conditions constitutes evidence for a pyrimidine ring with an amino group in the 2-position (165). The fluorescent

dibasic acid showed characteristic absorption spectra in 0.1N NaOH with maxima at 253 and 365 mμ. The empirical formula $C_7H_5N_5O_3$, the titration data, and formation of guanidine suggested a 2-amino purine or a 2-amino pteridine. The absorption spectra, however, eliminated the possibility of a purine because purines do not have absorption maxima above 300 mμ. Thus the available evidence pointed toward a 2-amino pteridine with an enolic and a carboxy group. With this evidence available, attempts were made to synthesize pteridine having these functional groups. The compound was identified as 2-amino-4-hydroxypteridine-6-carboxylic acid by comparison with the synthetic compound.

The monobasic fluorescent pigment produced by decarboxylation was identified as 2-amino-4-hydroxypteridine.

The structure of these two pteridines was established by the following series of reactions (166). Diethylmesoxalate was condensed with 2,4,5-triamino-6-hydroxypyrimidine to yield isoxanthopterin carboxylic acid (II) (167). The structure of isoxanthopterin carboxylic acid (II) is that shown by formula II although it had not previously been definitely established whether the carboxyl group occupied the 6- or 7-position. On chlorination of isoxanthopterin carboxylic acid and subsequent reduction with hydrogen iodide one of the hydroxyl groups was removed to give compound IV which was identical with the dibasic fluorescent pigment (166). Presumably either the 4 or the 7 hydroxyl could have been removed by this procedure. The presence of the 4 hydroxyl in compound IV was shown in two ways. First decarboxylation of 2-amino-4-hydroxypteridine-6-carboxylic acid (IV) gave 2-amino-4-hydroxypteridine (V), the structure of which was established by its synthesis from glyoxal and 2,4,5-triamino-6-hydroxypyrimidine (I). Its formation by this method demands a hydroxyl group in the 4 position. Second, the synthesis of 2-amino-4-hydroxypteridine-6-carboxylic acid was accomplished by condensation of 2,4,5-triamino-6-hydroxypyrimidine (I) and ethyl-β,β-diethoxy-α-bromo propionate. This reaction also establishes the presence of a hydroxyl group in the 4 position of the pteridine.

The final proof establishing the 6 position of the carboxyl group in compound IV was obtained by degrading 2-amino-4-hydroxy-6-methyl pteridine (VI) to give a compound identical with 2-amino-5-methyl-pyrazine (VII). The corresponding 7-methyl pteridine would have yielded 2-amino-6-methyl-pyrazine instead. The structure of 2-amino-4-hydroxy-6-methyl pteridine (VI) was established by oxidation with alkaline permanganate to give the corresponding 2-amino-4-hydroxypteridine-6-carboxylic acid (IV). These reactions are outlined in Fig. 2.

By hydrolysis with 0.5N sulfurous acid at room temperature pteroyltriglutamic acid was rapidly inactivated and gave an aromatic amine and a fluorescent pigment (168). This pigment reacted rapidly with typical aldehyde reagents such as hydroxylamine, phenylhydrazine and semicarbazide, indicating the presence of an aldehyde group. This fluorescent pigment did not possess a carboxyl group as evidenced by the fact that its distribution coefficient between water and buta-

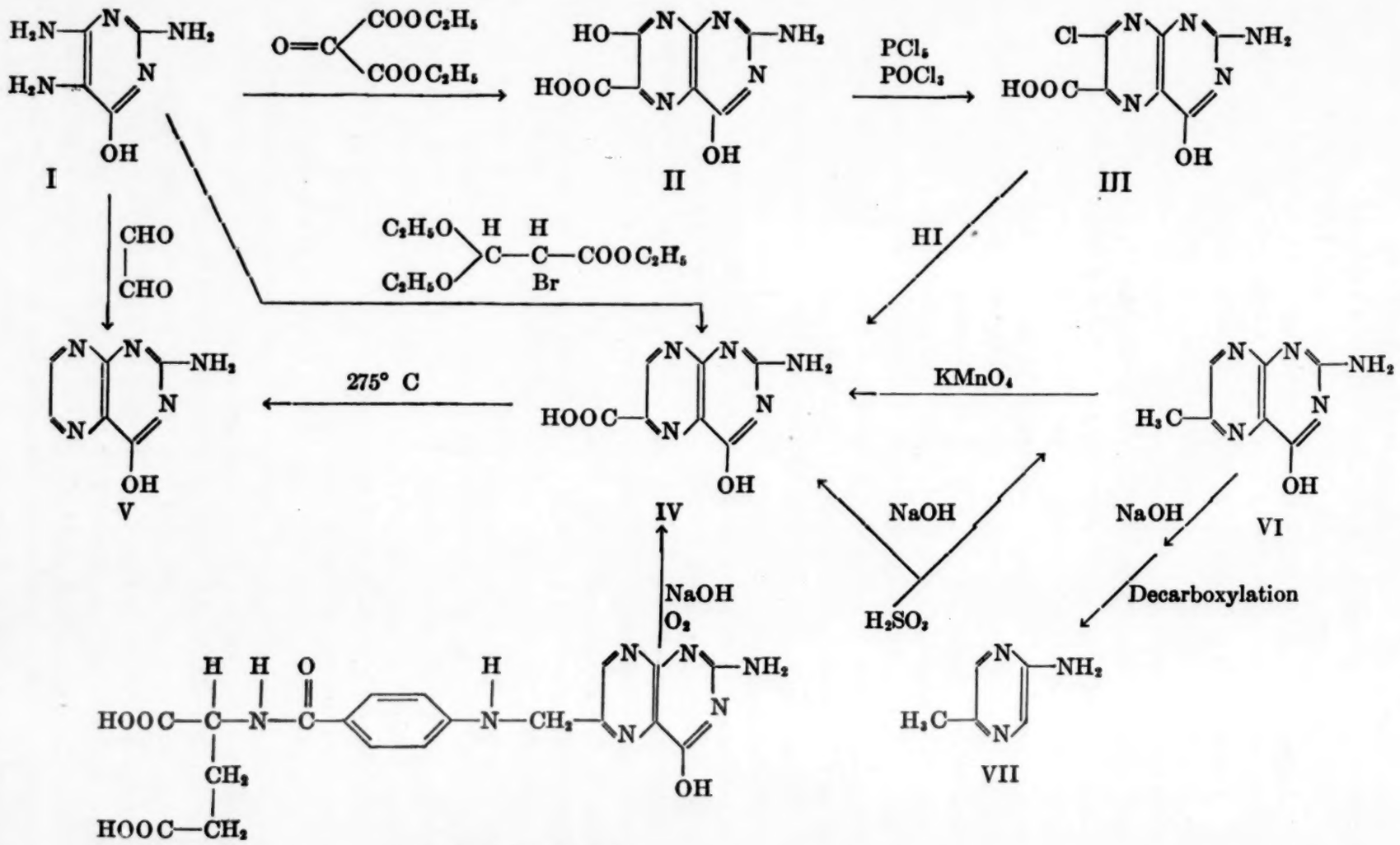

Fig. 2. Chemical reactions leading to the establishment of the structural formula of the pteridine (IV) obtained by aerobic alkaline hydrolysis of *L. casei* factor.

nol was the same at pH 3.0 as at pH 7.0. When the pigment obtained by sulfurous acid hydrolysis was treated anaerobically with dilute sodium hydroxide, approximately equal amounts of 2-amino-4-hydroxypteridine-6-carboxylic acid (IV) and a second pteridine, identified as 2-amino-4-hydroxy-6-methylpteridine (VI), were formed. The latter compound could be oxidized with alkaline potassium permanganate to yield 2-amino-4-hydroxypteridine-6-carboxylic acid (IV). The formation of approximately equal molal quantities of carboxy- and methyl-derivatives from what is apparently an aldehyde probably involves a Cannizzaro type reaction although the mechanism of this reaction is obscure.

Prolonged aqueous hydrolysis of pteroyltriglutamic acid at pH 4 produced biological inactivation and yielded a compound which was crystallized and identified as 1-pyrollidonecarboxylic acid (168). On hydrolysis with alkali this yielded 1 (+)-glutamic acid which was estimated microbiologically.

The aromatic amine which was produced during sulfurous acid hydrolysis was isolated as the barium salt (168). This compound when diazotized and coupled with N(1-naphthyl)ethylenediamine dihydrochloride yielded a red pigment (164) which indicated a primary aromatic amine with a highly negative substituent group. The aromatic amine nitrogen as measured by the method of Bratton and Marshall (164) constituted approximately 25 per cent of the total nitrogen. The remaining 75 per cent of the nitrogen could be converted into alpha-amino-acid nitrogen by alkaline hydrolysis. From such hydrolysates the aromatic amine was isolated and identified as *p*-aminobenzoic acid. Microbiological assay of the hydrolysate indicated the presence of 3 mols of glutamic acid. The peptide linkage of the glutamic acid to *p*-aminobenzoic acid must involve the carboxyl group of the latter as a primary aromatic amine is required for reaction in the Bratton and Marshall test (164).

The diazotizable aromatic amine obtained by aerobic alkaline hydrolysis of racemic pteroylglutamic acid was found to contain 2.1 atoms of nitrogen for each atom of aromatic amino nitrogen (163). The distribution coefficient of this aromatic amine was greatly different from that of p-aminobenzoic acid. After hydrolysis with 2N sulfuric acid, 45 per cent of the total nitrogen appeared as alpha-amino-acid nitrogen, and the distribution coefficient of the aromatic amine became the same as that for p-aminobenzoic acid. The latter compound was isolated from the hydrolysate.

Evidence regarding the mode of linkage is furnished by the results of alkaline hydrolysis (163). The absence of fluorescence and of the free aromatic amine in the original pteroylglutamic acid, and the simultaneous appearance of these two during aerobic alkaline hydrolysis suggested that the pteridine is linked to the aromatic amine nitrogen. As hydrolysis proceeded the liberation of pteridine and aromatic amine appeared at approximately the same rate.

Reduction in acid solution either catalytically or with zinc dust yielded the aromatic amine and a reduced pteridine (168). After reoxidation with manganese dioxide the pteridine obtained by zinc reduction was identified as 2-amino-4-hydroxy-6-methylpteridine, the structure of which has already been established.

The foregoing evidence indicated the following conclusions regarding the structure of pteroylglutamic acid.

1. Aerobic alkaline hydrolysis, sulfurous acid cleavage, and chemical or catalytic reduction each yielded a pteridine and a primary aromatic amine. This indicated the linkage of the pteridine to the nitrogen of the aromatic amine.

2. The aromatic amine formed during sulfurous acid cleavage of pteroyltriglutamic acid was a tetrapeptide, p-aminobenzoyldiglutamylglutamic acid. The aromatic amine from pteroylglutamic acid was p-aminobenzoylglutamic acid.

3. There was a single carbon atom linkage between the pteridine and the aromatic amine. This was indicated by the fact that only pteridines with a single carbon atom side chain were obtained and that no other two-carbon fragments could be detected in the two degradation reaction products. The evidence also indicated that this single carbon atom is present in a methylene link. If it were present as a CO group in an amide linkage the cleavage would be hydrolytic and would not require oxygen. The formation of 2-amino-4-hydroxy-6-methylpteridine also constituted evidence for the methylene linkage.

Synthesis. The final proof of the structure was obtained by synthesis of pteroylglutamic acid by different methods. One of these (101) involved the simultaneous condensation of 2-4-5-triamino-6-hydroxypyrimidine (I), p-aminobenzoylglutamic acid (IX) and α,β-dibromopropionaldehyde in aqueous solution. The other (169) involved the reaction of α,β-dibromopropionaldehyde with pyridine, 2,4,5-triamino-6-hydroxypyrimidine (I) and potassium iodide to yield N[(2-amino-4-hydroxy-6-pteridyl)methyl] pyridinium iodide (X). This was then treated with p-aminobenzoylglutamic acid (IX) in ethylene glycol to yield pteroylglutamic acid. The position of the methyl pyridinium group on the 6 position of the pteridine was established by oxidation with alkaline permanganate to yield 2-amino-4-hydroxypteridine-6-carboxylic acid (IV). These two methods of synthesis are schematically outlined in Fig. 3

Pteroic acid was obtained by the corresponding reaction (101) except that *p*-aminobenzoic acid was used instead of *p*-aminobenzoylglutamic acid.

Pteroylglutamic acid in the nutrition of mice. Indications that folic acid was required by mice on a purified diet containing sulfasuxidine were reported (170). The "lactation performance" of mice on purified diets as measured by the percentage and size of litters weaned was increased by adding a concentrate containing pteroylglutamic acid (171, 172).

Dogs. Observations with dogs (173) and pigs (174) indicated that an unidentified B complex factor or factors present in liver and yeast, were needed as a supplement with purified diets. It seems probable that the liver and yeast extracts used in these investigations supplied pteroylglutamic acid. Pteroylglutamic acid was reported to improve the response to niacin in dogs on purified diets deficient in niacin (175).

Guinea pigs. A series of studies with guinea pigs on purified diets indicated that three unidentified dietary factors were required, two of which were found present in linseed oil meal. It was later shown that pteroylglutamic acid could

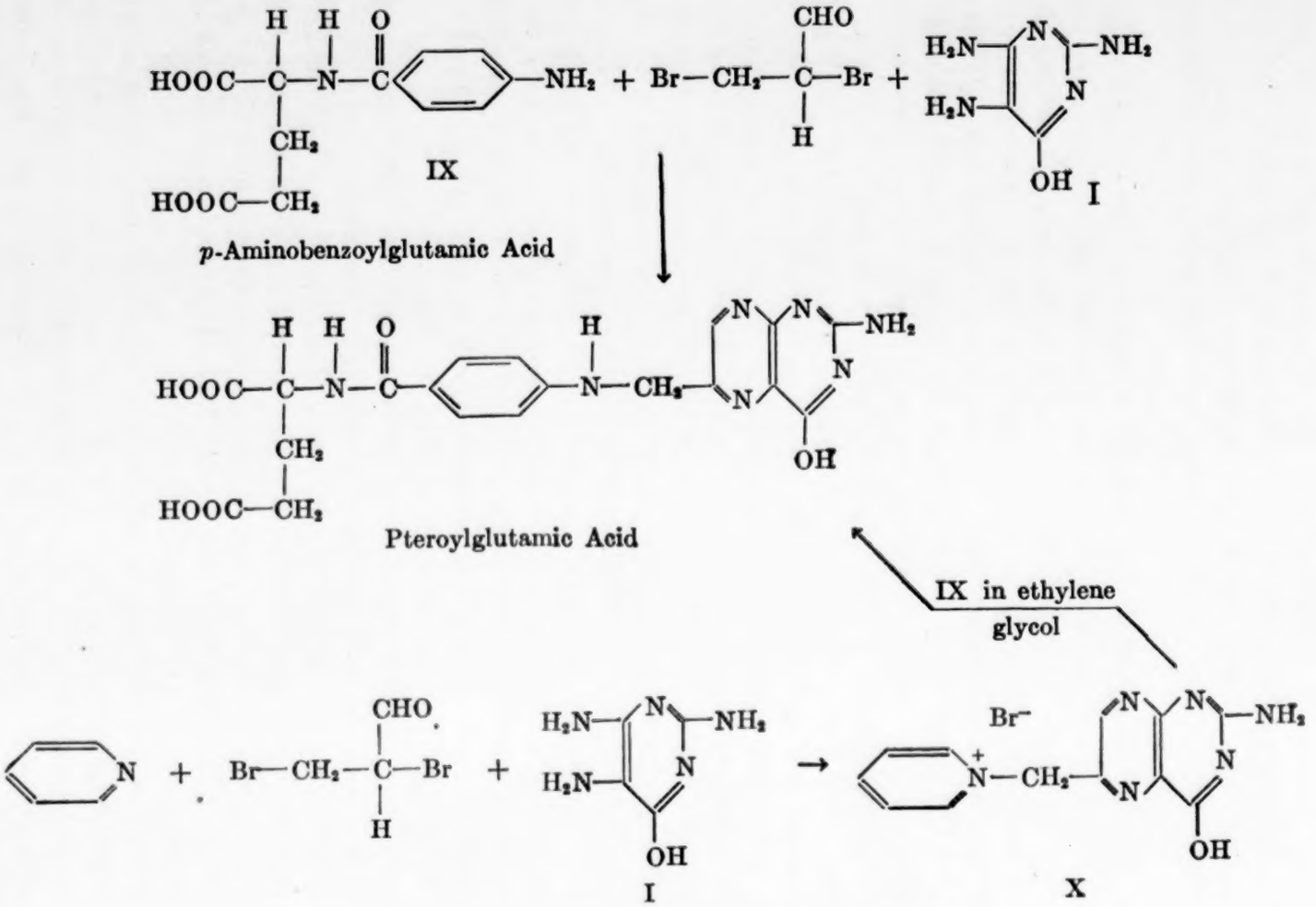

Fig. 3. Two syntheses of pteroylglutamic acid.

replace one of the factors (176). The deficiency was described as being characterized by a rapid decline in weight, lethargy, salivation, terminal convulsions and death. The daily administration of 6.5 micrograms of either pteroylglutamic acid or pteroyltriglutamic acid prevented the deficiency.

Nutrition of insects. Larvae of the mosquito *Aedes aegyptii* were found to need concentrates of pteroylglutamic acid for pupation; xanthopterin or thymine were ineffective. Pteroylglutamic acid was also found to promote growth and to increase survival rate (177). It was found that the larvae of the flour moth and the meal worm needed pteroylglutamic acid for growth (178). Growth of the larvae of a carpet beetle was increased by concentrates of pteroylglutamic acid (179).

Mink. The effects of pteroylglutamic acid deficiency in mink were found to include loss of body weight, diarrhea, irritability, general weakness, anorexia and leukopenia. The deficiency responded to administration of pteroylglutamic acid. There was some indication that an unidentified factor in liver was also needed (180).

Pigs. It was suggested that pteroylglutamic acid deficiency in pigs might be associated with the development of normocytic anemia, although no actual test with pteroylglutamic acid was carried out (181). The pigs from sows fed a supplementary "folic acid concentrate" were thriftier and more vigorous than were pigs from sows fed the basal diet of corn and soy bean meal supplemented with vitamins, not including pteroylglutamic acid, and minerals (182). It was reported that pteroylglutamic acid "may have helped slightly with hemoglobin formation" in pigs on a purified diet (183). Other observations (174) were mentioned above.

The enzymic liberation of pteroylglutamic acid from its "conjugated" forms. The effect of enzymic action in liberating folic acid from tissues was indicated by an observation that the apparent "folic acid" content of such materials could be increased several-fold by treatment with a crude enzyme-containing preparation ("taka-diastase") (184). It was found possible to prepare a dialyzable fraction from yeast which was active for the "vitamin B_c" deficient chick but which had relatively little potency in stimulating the growth of *L. casei* (92) until the concentrate was subjected to an undescribed "enzymatic digestion" which liberated "vitamin B_c" in a microbiologically active form.

Almost at the same time, it was noted that yeast was "rich in the substances which give rise to folic acid when incubated with fresh liver" (30). A crude enzymically-active preparation was made from rat liver (36) by extraction with phosphate buffer, fractional precipitation with ammonium sulfate, and dialysis. The preparation liberated the *S. lactis* R-stimulating factor at pH 7 from cell-free water-soluble fractions. These fractions had previously been extracted from natural materials with the aid of "taka-diastase" which thus had brought pteroylglutamic acid conjugates into solution without splitting them. Hence taka-diastase did not supply appreciable quantities of the "folic-acid-liberating" enzyme system. This observation indicated a degree of specificity on the part of the enzyme concentrate prepared from rat liver. The name "vitamin B_c

conjugase" was applied to the enzyme system (185). Hog kidney, liver, small intestine and beef liver were reported to be rich sources; it also occurred in sweet almonds. The optimum pH of the hog kidney preparation was 4.5, while that for almond was 7.0. Hog kidney was used as a source of the enzyme in a method for the assay of "vitamin B_c conjugate" (186). Samples were incubated for 16 hours at pH 4.5 and 45°. The values obtained after enzymatic treatment in many cases showed close agreement with the values obtained by biological assay with chicks on a purified diet (68). An exception was observed in the case of certain liver extracts and plant extracts. The presence of inhibitors in the plant extracts was suggested, or, as an alternative, the presence of compounds which were active for the chick but inactive for *L. casei* even after treatment with the enzyme.

Chicken pancreas (187) was found to be an excellent source. An increase in potency of more than one-thousand fold was reported to occur upon concentrating the enzyme from chicken pancreas extract by adsorption and precipitation techniques. The optimum pH for the action of the enzyme was between 7 and 8; a considerable loss of activity was observed on dialysis. Further studies (158) described modifications in the procedure for concentrating the enzyme. The pancreatic tissue was ground and allowed to autolyze at pH 8. A fraction was then separated which precipitated at between 40 and 80 per cent saturation of ammonium sulfate. The precipitate was dialyzed, redissolved, reprecipitated with cold alcohol, redissolved in buffer and reprecipitated with ammonium sulfate. The procedure resulted in an approximately 3000-fold concentration of activity. The concentrated preparation was activated by calcium, the optimal concentration being 0.01M. Optimum pH and temperature were found to be 7.8 and 32°.

The Michaelis constant for conjugase preparations from rat liver, potatoes and chicken pancreas was measured (188).

The production of "vitamin B_c activity" in several hundred different microorganisms was measured by *L. casei* assay. Various commercial enzymic preparations were tested for their activity in liberating pteroylglutamic acid from its conjugates but none of the preparations were very effective. Chicken pancreas was used as a source of the enzyme (189).

A study was made of the comparative potencies of various tissues in liberating ptercylglutamic acid from a concentrated preparation of pteroylheptaglutamic acid (vitamin B_c conjugate) (190). Chicken and turkey pancreas were the most potent tissues; their optimum activity was at a pH of about 7.0 while other tissues; including rat, mouse, hog and guinea pig pancreas, chicken and hog liver and hog kidney had their optimum at a pH of about 4.5. Hog kidney was used as a source of the "conjugase" enzyme system for further studies. The optimum temperature for the action of the enzyme was found to be 45° to 48°. The presence of an inhibitor in yeast extract was demonstrated. Loss of activity occurred during attempts to concentrate the conjugase; the addition of calcium failed to restore the loss. A unit of activity for the enzyme was proposed and defined.

The p-aminobenzoyl-polyglutamic-acid polypeptide isolated from yeast (191)

was found under certain circumstances to inhibit "vitamin B_c conjugase" preparations obtained from chicken pancreas and rat liver (Totter, J. R. Cited in (192)).

Clinical effects of pteroylglutamic acid. It is beyond the scope of this review to discuss the extensive literature of recent years regarding the erythrocyte maturation factor present in liver and the extrinsic and intrinsic factors which have been postulated to be concerned in its formation. Mention must be made, however, of the baffling circumstances that simultaneously relate and contrast pteroylglutamic acid and the erythrocyte maturation factor. Both are present in liver, and both will produce hematologic remission of addisonian pernicious anemia. However, the processes commercially used in the refinement of concentrated solutions of the erythrocyte maturation factor do not result in the concentration of pteroylglutamic acid, in fact the original pteroylglutamic acid content of crude liver extracts is largely diverted into side fractions during the process. Experimental animals, even when they are acutely deficient in pteroylglutamic acid, do not respond to preparations of the erythrocyte maturation factor. In the treatment of pernicious anemia, the erythrocyte maturation factor is far more effective when injected than when fed to pernicious anemia patients, while pteroylglutamic acid is approximately equally effective by either route of administration. Differentiation of pteroylglutamic acid from the "extrinsic factor" appears to be established by the activity of pteroylglutamic acid when fed or injected in producing hemopoietic remission of pernicious anemia without the addition of normal human gastric juice.

The results of various clinical experiments, most of which did not permit of definite conclusions and which were made with crude materials or natural concentrates, were published prior to the availability of pteroylglutamic acid. The investigations of Wills were mentioned on p. 54; these findings served to differentiate the erythrocyte maturation factor from an unidentified factor in yeast and liver which was effective against tropical macrocytic anemia and against a corresponding anemia in monkeys. Recently this question was reopened (193) in an article which drew attention to preceding observations (194, 195, 196, 197, 198, 199, 200) that certain macrocytic anemias usually associated with pregnancy and encountered both in the tropics and the temperate zone did not respond to the injection of liver extracts which were effective against pernicious anemia but these anemias responded to the oral administration of liver or autolyzed yeast. Three cases with histories of "striking dietary inadequacy" were found to respond to oral liver extract after no response had occurred to smaller volumes of injected liver extract.

Five patients with pernicious anemia were found to respond to comparatively large doses of brewers' yeast 1 to 2 grams per kilo of body weight daily without the addition of gastric juice (201). One may speculate that this response was probably due to pteroylglutamic acid.

A concentrate of "vitamin B_c" from yeast, standardized by chick assay, was fed to ten patients with refractory macrocytic anemias. A dosage rate corresponding to 0.6 mgm. of pteroylglutamic acid daily was used for the first week,

and it was increased to 1.5 mgm. daily for the next 3 weeks. With the exception of an increase in the hematocrit reading, no significant changes were obtained in the blood picture (202).

Negative results were obtained in the treatment of 2 cases of pernicious anemia with pteroyltriglutamic acid 3.6 or 2.3 mgm. daily by mouth for 10 days (203). This may be contrasted with the subsequent report of a single case which responded to pteroyltriglutamic acid, 3 mgm. daily, by injection (204).

As a result of the preceding and other studies (16, 193, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214) it became evident by 1945 that there existed a nutritional macrocytic anemia often associated with pregnancy, which responded to the feeding of crude liver extracts, yeast or yeast extracts, but which did not respond to the injection of potent sources of the anti-pernicious-anemia factor of liver extracts. The anemia was characterized in some cases (215) by a history of marked dietary inadequacy especially with respect to meat; the presence of gastric hydrochloric acid; the absence of neural manifestations; and the presence of a megaloblastic bone marrow. Maintenance therapy with liver was not required. A similar condition was encountered which responded to the injection of concentrated liver extracts (193, 194, 216, 217, 218, 219); the differentiation of this from the preceding type of anemia appeared to be possible only on the basis of response to such extracts. The time was now ripe for the appearance of a new therapeutic substance. This substance proved to be pteroylglutamic acid.

In the summer of 1945, synthetic pteroylglutamic acid was made available in fairly large quantities for clinical experiments. Results were soon obtained and reported in the literature; it was apparent that the use of adequate dosage schedules led to the establishment of positive responses to pteroylglutamic acid in the treatment of anemias which were accompanied by megaloblastic erythropoiesis.

Addisonian pernicious anemia. Descriptions of the response of this disease to pteroylglutamic acid first appeared in December, 1945 (22, 220).

The effects of pteroylglutamic acid on 14 cases of macrocytic anemia in relapse were described (22). Five of these cases were classified as addisonian pernicious anemia, five as nutritional macrocytic anemia, and two as indeterminate. The patients received pteroylglutamic acid, 20 mgm. to 50 mgm. injected or 100 mgm. by mouth daily. One patient received 150 mgm. by mouth. A feeling of subjective improvement occurred between the third and fifth days and was accompanied by an increase in appetite, which was in some cases associated with "remarkable weight gains", in most patients. The initial red blood cell count varied from 1.64 million to 2.97 million cells per cu. mm. Except in the case of one patient who did not respond, the final red cell count at 18 to 55 days varied from 2.92 to 4.29 million cells per cu. mm. Oral administration produced greater reticulocyte responses and more rapid regeneration of blood than did parenteral administration. However, the oral dosage was usually 5 times as great as the parenteral. Increases were also noted in per cent hemoglobin and in total white cell count. Reticulocyte crises were observed in from 3 to 10 days.

The administration of pteroylglutamic acid was continued for periods up to 30 days and the red cell count and the per cent hemoglobin continued to rise after the treatment was discontinued.

In the other study (220) two patients with pernicious anemia were studied. The first patient with pernicious anemia was given 100 mgm. of pteroylglutamic acid orally each day for 10 days. Her initial red blood cell level was approximately 1.2 million cells per cu. mm. On the third day of therapy she experienced a feeling of well-being and increased appetite and on the next day her reticulocytes began to rise and a peak value of 40 per cent was reached on the seventh day. Her red blood cells began to increase in number on about the seventh day and the count rose rapidly until a level slightly over 3 million was reached. The second patient responded in a similar manner.

The use of pteroylglutamic acid in a case of untreated addisonian pernicious anemia was described (221). The patient received 2.0 mg. of pteroylglutamic acid intravenously daily for 20 consecutive days. The red cell count before treatment was 1,490,000 per cu. mm. with 5.8 grams of hemoglobin. Microscopic examination of the bone marrow showed a picture typical of a deficiency of the maturation factor. After treatment "daily bone marrow examinations demonstrated a gradual megaloblastic maturation with the marrow picture approaching normal on the tenth day of therapy." The maximum reticulocyte response (26.8 per cent) was obtained on the fifteenth day of therapy, after a total cumulative dosage of 30 mgm. to see if a secondary reticulocyte rise could be induced. This was not observed. On the fortieth day of therapy the red cells reached 4,000,000 per cu. mm. and the hemoglobin 13.0 grams. There had been a definite corresponding rise in total white cells and platelets. The same publication described the effects of pteroylglutamic acid in three patients sensitive to liver extract. In each instance, the local reaction to the injection of liver extracts was extremely marked, and in no instance was there any reaction to pteroylglutamic acid greater than to normal saline.

In another report (222) hemopoietic responses to pteroylglutamic acid in 5 cases of pernicious anemia were described after daily dosage with 20 mgm. of pteroylglutamic acid intravenously or 100 mgm. by mouth. Increases in erythrocyte counts and hemoglobin levels were observed, together with reticulocyte crises ranging from 6.4 to 14.8 per cent. Positive responses to pteroylglutamic acid in the treatment of 4 cases of pernicious anemia were described (223). Results similar to the preceding were noted (23), and it was found that one patient with pernicious anemia had neurologic changes which improved with pteroylglutamic acid therapy, although complete return to normal had not been obtained at the time of writing.

The responses of 6 patients with addisonian pernicious anemia to pteroylglutamic acid were described (224). Clinical improvement and hematological remission were noted in all cases. In one case muscular weakness and loss of vibratory sense of both lower extremities were observed; these findings were unchanged after 79 days of treatment with pteroylglutamic acid, 25 mgm. to 100 mgm. daily.

Other reports (225, 226, 227) described hematological responses to pteroylglutamic acid in pernicious anemia patients.

Studies were reported (227) on the response of various macrocytic anemias. Macrocytosis alone did not necessarily imply either megaloblastic erythropoiesis in the marrow nor responsiveness to pteroylglutamic acid. It was considered justifiable to regard megaloblastic erythropoiesis as the morphologic expression of pteroylglutamic acid deficiency. The authors studied 11 cases of macrycotic anemia treated with pteroylglutamic acid. Five of these were pernicious anemia cases which responded promptly. The remaining six cases, all of which showed normoblastic marrow patterns, did not respond; these included four cases of portal cirrhosis, one of acute infectious hepatitis (Weil's disease) and one of subacute lymphatic leukemia.

Pteroyltriglutamic acid. A patient with pernicious anemia in relapse was treated with pteroyltriglutamic acid, 3 mgm. daily, by intramuscular injection for 11 days. A submaximal hemopoietic response was observed, accompanied by subjective improvement (204). It may be presumed that a more marked response could have been obtained with a higher dosage.

Thymine. Observations of the growth-promoting effects of this substance on *L. casei* and *S. fecalis* R (121, 103, 105) were followed by studies of its effect in pernicious anemia. Large amounts were necessary to produce an effect; the administration of 1 gram or less gave no response in pernicious anemia, sprue or nutritional macrocytic anemia (228). One case of pernicious anemia responded to 6 grams daily by mouth for 14 days; three cases to doses up to 3.4 grams daily for 11 days (229) and six cases to doses of 4.5 to 10.2 grams daily (230). The necessary amounts appeared to be in the neighborhood of 1000 times as great as the adequate dosage of pteroylglutamic acid. A similar ratio of effective dosage between thymine and pteroylglutamic acid was observed with the lactic acid bacteria (see p. 65).

Combined system disease. By the summer of 1946 it had been well established that pteroylglutamic acid would consistently produce a prompt and satisfactory hemopoietic response in addisonian pernicious anemia. A long period of study was necessary to compare the effects of liver extract and pteroylglutamic acid in preventing the onset of neurological signs and symptoms in pernicious anemia.

The results of a 12-month investigation of this point were reported (231). Twenty-six patients were studied, 21 of whom had pernicious anemia which had been controlled for from 2 to 17 years by injections of liver extract. Three had pernicious anemia in relapse and two had sprue which had been poorly controlled by liver extract. Liver therapy was discontinued and the patients were treated with 70 to 105 mgm. pteroylglutamic acid per week in divided doses given orally for 10 to 12 months. Most of the patients noted an increase in appetite and weight. In two instances there were moderate increases in the hematological values after 5 to 8 months which increased until there was evidence of combined system disease. The dosage of pteroylglutamic acid was increased without signs of improvement, following which refined liver extract, 5 cc. daily,

was administered and neurological improvement was reported to be observed in 10 days.

In another study (232) the following data were obtained regarding the effect of pteroylglutamic acid on 14 cases of pernicious anemia.

	GLOSSITIS	PARESTHESIA	COMBINED SCLEROSIS
Before treatment			
	7	10	2
During treatment			
Improvement	3	4	
Temporary improvement	3	4	
No improvement	1	2	2
New manifestations		2	3

The effect of pteroylglutamic acid on pernicious anemia and combined system disease was studied in a number of patients (233). Three patients with neurological involvement received pteroylglutamic acid, 25 to 50 mgm. by mouth or 20 mgm. intramuscularly daily. The patients showed hematological responses but their neurological symptoms continued to progress. Administration of liver extract resulted in improvement in the signs of nervous system changes. Other pernicious anemia patients in relapse received 0.5 unit of liver extract with 5 to 10 mgm. of pteroylglutamic acid daily and showed reticulocyte responses which were greater than those anticipated as a result of previous experience with liver alone.

Rapidly progressive neurological relapse was observed in a patient with pernicious anemia who had been maintained on pteroylglutamic acid for 12 weeks. The diet during this period was poor. The patient subsequently improved during treatment with liver extract. The patient was one of a group of 47 patients with pernicious anemia who were maintained with pteroylglutamic acid for periods up to one year. Only two others showed neurological relapse. Both of these had a poor dietary history. They showed mild symptoms which responded readily to liver extract (234).

These preliminary studies indicated that the erythrocyte maturation factor, or some unidentified substance which accompanies it in therapeutic amounts in refined liver extract, is needed for the prevention of neurological signs and symptoms in certain cases of combined system disease and that pteroylglutamic acid is not consistently effective in this regard. It would be of interest to study the effects of pteroylglutamic acid upon the incidence of combined system disease when administered together with large amounts of the known B complex vitamins.

Possible relation of pteroylglutamic acid to the biochemical defect in pernicious anemia. A disturbance in the utilization of pteroylglutamic acid appears to

occur in pernicious anemia. This is evidenced by the following considerations:

a. In contrast to certain other anemias, pernicious anemia has never been associated with an obviously incomplete diet.

b. Dosage with pteroylglutamic acid, usually in amounts which are greater than those occurring in ordinary diets, will produce a hemopoietic response in pernicious anemia.

c. Certain cases of pernicious anemia do not respond to crude concentrates of pteroylheptaglutamic acid. This finding is discussed below.

Preliminary publications speculated upon the possibility that a basic defect in pernicious anemia is a failure to liberate pteroylglutamic acid from its conjugates (235, 236). It was observed (236) that when pteroylheptaglutamic acid (vitamin B_c conjugate) was administered to normal individuals there was increased urinary excretion of pteroylglutamic acid. However, no such increase was observed when pteroylheptaglutamic acid was administered to pernicious anemia patients (235, 236). It was therefore portulated that in these patients a pteroylglutamic acid deficiency resulted from the imperfect utilization of conjugated forms of pteroylglutamic acid and that as a result the supply of pteroylglutamic acid was insufficient for the maintenance of the hemopoietic mechanism. Three patients with pernicious anemia and one with macrocytic anemia following gastrectomy showed no evidence of a therapeutic effect from the administration of pteroylheptaglutamic acid, equivalent to 2.3 to 4 mgm. of pteroylglutamic acid daily for 8 to 12 days. When equivalent amounts of pteroylglutamic acid were substituted for the conjugate all of the patients showed significant clinical and hematologic responses (235). It was reported (236) that the daily administration of 1 mgm. of "yeast conjugate", presumably pteroylheptaglutamic acid, for 10 days to a patient with pernicious anemia in relapse produced no response. During a second period of 11 days, 100 cc. of normal human gastric juice was administered with the yeast conjugate, again without response. The patient then responded submaximally to a daily dose of 0.35 mgm. of pteroylglutamic acid. A second patient received intramuscular injections of 2.5 mgm. of yeast conjugate daily for 12 days, followed by a single injection of 30 mgm. of conjugate, without responding. After a further 12 days, administration of pteroylglutamic acid causes a theoretically maximal reticulocyte response. In two patients with pernicious anemia, injection of concentrated liver extract containing insignificant amounts of pteroylglutamic acid doubled the urinary excretion of pteroylglutamic acid. This observation fitted the interpretation that a constituent of liver extract might be concerned with the activation of a "conjugase system", or with the removal of an inhibitor of such a system (237) but it should be noted that the urinary excretion of pteroylglutamic acid on a normal diet is extremely small both in normal subjects and in pernicious anemia patients, so that doubling the urinary excretion of pteroylglutamic acid is quantitatively only a small increase.

In another report (238) earlier findings (235) were extended. The heptaglutamate was given orally in the form of a concentrate to nine cases of pernicious anemia in relapse, three in remission induced by liver extract, two with macro-

cytic anemia following gastrectomy, and six healthy subjects, without effect on the anemias, all of which subsequently responded to pteroylglutamic acid. No pteroylheptaglutamic acid was found present in the urine in any of the experiments. The patients excreted variable amounts of pteroylglutamic acid following its administration while the amounts excreted by the normal subjects were "considerably higher and more constant". After the administration of pteroylheptaglutamic acid the patients with one exception showed no significant increase in excretion of pteroylglutamic acid, but when pteroylheptaglutamic was administered to the patients in remission induced by liver extract, an excretion of pteroylglutamic acid in amounts similar to those observed with healthy subjects was found. This suggested that "the principle of liver active in pernicious anemia may be concerned with the conversion of the conjugated vitamin to the free form".

More detailed findings were subsequently reported (239) and the earlier postulations regarding the function of the erythrocyte maturation factor were modified in the light of these results. Emphasis was laid upon the role of "conjugase inhibitors", as present in certain yeast concentrates, in modifying the utilization of "hexaglutamyl conjugate" (pteroylheptaglutamic acid) by pernicious anemia patients. The urinary excretion of pteroylglutamic acid after the administration of conjugate with "large amounts of inhibitor" was much less in pernicious anemia patients than in normal subjects. Two patients, one in relapse and one in partial remission, were treated with conjugate *plus* liver extract without markedly increasing the urinary pteroylglutamic acid excretion over the levels observed when the conjugate was given alone. In contrast, three patients in remission who received the conjugate excreted pteroylglutamic acid in amounts approximately equal to those observed in normal subjects. It was concluded that the diminished ability of pernicious anemia patients to utilize the conjugate was not absolute, varied in different patients, and was partly dependent upon a conjugase inhibitor present in natural materials. The effect of the inhibitor was studied in normal persons (240), and its administration was found markedly to reduce the urinary excretion of pteroylglutamic acid following the feeding of conjugate, 4 mgm. daily. Attention was drawn to the high inhibitor content of liver, yeast and spinach. The conjugate was not found present in the urine in any of the experiments.

Others have also pointed out that certain pernicious anemia patients are able to utilize concentrates of the conjugates (192). It is difficult in the present state of knowledge to generalize regarding the biochemical defects in pernicious anemia. It appears to be evident that there is an abnormality in the utilization of pteroylglutamic acid. The variability in the findings may be due to variations in the extent and type of the biochemical lesions in the patients.

Another basis for possible speculation lies in some observations of the excretion of xanthopterin (47). It was reported that the normal daily excretion of xanthopterin in the urine was increased by approximately 50 per cent in untreated pernicious anemia. The excretion dropped to normal in 11 of 13 patients after remission had been brought about by liver extract. If pteroyl-

glutamic acid is broken down to xanthopterin and is excreted as such in the urine, these observations might imply that there is an increased rate of breakdown of pteroylglutamic acid in pernicious anemia. However, two other patients in this study had extremely low urinary xanthopterin values which increased after treatment with liver extract.

Sprue. The successful treatment of sprue with pteroylglutamic acid was first reported in November 1945 (241). Two patients were treated daily with 15 mgm. of pteroylglutamic acid by intramuscular injection. The symptoms of glossitis disappeared after 4 days treatment in the first case. After nine days the reticulocytes reached a peak of 15.3 per cent and a marked increase in thrombocytes was noted. The red cell count rose from 1.56 to 3.88 cells per cu. mm. and the per cent hemoglobin rose from 6.0 to 9.5. These changes were accompanied by marked general betterment, including regeneration of the lingual papillae, subsidence of the diarrhea and considerable gain in weight. The tolerance curves for oral glucose and vitamin A became more nearly normal. The second case received similar treatment and showed a reticulocyte crisis on the 4th day accompanied by subjective improvement.

In another report (220) 20 mgm. of pteroylglutamic acid was injected intravenously each day for 10 days to a patient with sprue. Following the initial ten-day period 40 mg. was injected every other day for an additional two weeks. A reticulocyte peak of 30.2 per cent was obtained on the seventh day although the initial red count was 2.6 million cells, so that the reticulocyte response was greater than was anticipated. The patient gained 8 pounds within $3\frac{1}{2}$ weeks and the oral glucose tolerance test showed much better absorption.

Three cases of "tropical" sprue were treated with 200 mgm. of pteroylglutamic acid daily by mouth (242). Subjective improvement was noted after 3 or 4 days. The erythrocyte count was 1.15 to 2.16 million cells per cu. mm. on the 10th day. Reticulocyte crisis occurred in 6 to 7 days with peak values of 17 per cent to 22 per cent. Gains in strength, vigor and appetite were noted.

Further laboratory findings regarding patients with sprue were reported (243). Three patients were studied who fulfilled all of the criteria necessary for the diagnosis of sprue including glossitis, diarrhea with increased fat content of the stools, marked loss of weight, pigmentation of the skin, macrocytic anemia, moderate leucopenia, impairment of absorption as indicated by a flat oral glucose tolerance curve with a normal intravenous tolerance curve, a flat vitamin A tolerance curve and a very low serum carotene content, and a characteristic gastrointestinal pattern on x-ray examination. Free hydrochloric acid was present in the gastric juice of each. Sternal marrow of two of the patients was examined and found to be typical of that seen in untreated sprue and pernicious anemia. After a preliminary period of observation these patients received daily intramuscular injections of 15 mgm. of pteroylglutamic acid as the sole therapeutic agent. In all three cases prompt hematological and clinical improvement was noted including disappearance of glossitis within 3 or 4 days, regeneration of the lingual papillae, an improved sense of well-being, subsidence of diarrhea, improvement in appetite and gain in weight. One patient gained 26 pounds

within 6 weeks following the institution of treatment with pteroylglutamic acid. Reticulocyte crises were noted in 6 to 8 days together with increases in the number of platelets, the number of white blood cells, the number of red blood cells and the percentage of hemoglobin. Examination of aspirated sternal marrow after treatment showed the disappearance of the more primitive red blood cells and return of the white cell series to normal proportions.

Other reports with sprue have indicated comparable findings (23).

The effect of pteroylglutamic acid on plasma tocopherol levels in sprue was reported (244). The level gradually decreased to a very low value in one patient during a relapse following the withholding of pteroylglutamic acid therapy and increased following readministration of pteroylglutamic acid. During relapse it was observed that the patient showed a maximum rise in serum concentration of tocopherols of 0.09 mgm. per 100 cc. after an oral dose of 600 mg. of mixed tocopherols in contrast to an average peak of 0.37 mgm. in seven healthy adults after a similar dose. Attention was drawn to findings elsewhere that muscle tissue at autopsy from patients with sprue showed pigment resembling that seen in vitamin E deficient animals and that tocopherol deficiency has been observed in rats on purified diets containing sulfonamides.

Findings regarding the metabolism of pteroylglutamic acid and its conjugates were obtained in studies with two sprue patients (192). The first patient had been treated irregularly and inadequately with liver extract. He responded promptly to intramuscular injections of pteroyltriglutamic acid, 4.9 mgm. daily. After 10 days, concentrated liver extract was also administered, and the patient's recovery continued uneventfully. Microbiological assays of the urine showed that the amount of pteroylglutamic acid excreted daily before the initiation of treatment was so low as to be undetectable. Following the administration of pteroyltriglutamic acid, the urinary excretion of pteroylglutamic acid rose to a maximum of 3.66 mgm. on the sixth day, then declined to 1.34 mgm. on the tenth day. Upon starting liver extract which contained insignificant amounts of pteroylglutamic acid, the excretion rose to the remarkably high level of over 7 mgs. of pteroylglutamic acid daily for two days, in spite of the fact that the daily intake of pteroyltriglutamic acid corresponded to only 3.1 mgm. of pteroylglutamic acid. This finding indicated that some factor in liver extract had a marked effect on pteroylglutamic acid metabolism in sprue. The second patient was treated orally with a concentrate of pteroylheptaglutamic acid containing 8.4 mgm. of pteroylheptaglutamic acid and about 0.3 mgm. of free pteroylglutamic acid per daily dose. Remission promptly occurred with urinary excretion of only small amounts of pteroylglutamic acid. The excretion rose to a maximum of 0.32 mgm. on the tenth day, at which point the injection of liver extract was started, following which the urinary excretion continued to increase to a maximum of 1.5 mgm. Treatment of the urine of either patient with "conjugase" indicated the absence of conjugates. The number of cases is so small that the results until confirmed should be treated with reservation in spite of their great biochemical interest.

A patient with sprue was treated with 5 mgm. of pteroyltriglutamic acid twice

daily by intramuscular injection. A total quantity of 83 mgm. was given over a period of 9 days. A reticulocyte peak of 38 per cent occurred on the fifth day of therapy and was accompanied by a rise in erythrocyte count and in hemoglobin and by clinical improvement (245).

Thymine in sprue. Clinical and hematological improvement were reported in four patients with sprue who received 15 grams of thymine daily (246). Reticulocyte peaks were reached on the eighth or ninth day, and were followed by increase in erythrocytes and hemoglobin, subjective improvement and a return of the stools toward normal. The authors observed that the response was less dramatic than that observed with pteroylglutamic acid.

Nutritional macrocytic anemia. This condition was discussed and reviewed (17). In December 1945 (22), a consistent effect of pteroylglutamic acid in producing remission of 9 cases of nutritional macrocytic anemia was described. The responses were similar to those described for pernicious anemia in the same article (see p. 82). Additional details were given in a later publication (222). Similar results were obtained elsewhere (23). "Nutritional macrocytic anemia" occurring in a patient who had previously responded to pteroylglutamic acid and who had subsequently relapsed, was found to respond to pteroylheptaglutamic acid, 14 mg. daily for 9 days (247). The clinical improvement was rapid and was comparable to that observed with pteroylglutamic acid. A case of nutritional macrocytic anemia did not respond to pteroic acid, 5 mgm. by intramuscular injection daily for 8 days. The patient subsequently responded to dosage with pteroylglutamic acid, 5 mgm. daily.

Macrocytic anemia of pregnancy. A patient was described as having a red blood cell count of 1.1 to 1.2 million red blood cells per cu. mm. 18 days after parturition. She received 20 mgm. of pteroylglutamic acid intramuscularly for each of 10 days. Her subjective improvement was marked on the third day, and a peak value of 48 per cent reticulocytes was reached on the seventh day. The red cell count rose quite rapidly (220). Another report (24) described a response to pteroylglutamic acid in what was presumably a case of macrocytic anemia of pregnancy.

Megaloblastic anemia in infancy. A series of studies (248, 249, 250) described this condition as being characterized by:

1. Normochromic anemia, usually but not invariably macrocytic
2. A tendency toward leukopenia and neutropenia
3. A diminution of platelets, often associated with an increased bleeding tendency
4. A megaloblastic bone marrow pattern resembling or identical with the pattern seen in pernicious anemia in relapse
5. A frequent incidence of splenomegaly; an evidence of infection usually present; a histamine refractory achlorhydria present but reversible.

The treatment of 29 patients was described, 12 of these received pteroylglutamic acid either synthetic or in the form of a natural concentrate prepared by eluting a charcoal adsorbate of liver extract. The dosage rate was 5 to 20 mgm. per day for 8 days to 3 weeks. Three of the patients died with severe

infections. The remaining 9 responded, showing reticulocyte peaks, a return of the bone marrow pattern to normal, and increases in hemoglobin and red cell counts. Increases in the platelet counts were noted in blood smears at around the ninth day. The effect of pteroylglutamic acid was stated to be indistinguishable from that produced by liver extract and no relapses were observed in follow-up studies which lasted up to 10 months. The etiology of the condition, which was commonly associated with coughs and coryza, was indefinite. Of interest is the observation that one child had taken nothing but goats' milk for four months. This recalls the early observations with rats (44, 46). Another early observation (251) described the response of "goats' milk anemia" in infants to liver extract and to yeast. No response was obtained to iron.

Celiac disease. The occurrence of macrocytic anemia in celiac patients has been stated to be "exceedingly rare" (252). Results with the use of pteroylglutamic acid in the U. S. A. have not been encouraging (253). However, two reports have appeared in England; in one of these (254) the case of a 17-month old boy was described. The patient was emaciated and anemic. He had responded previously to treatment with liver extract, but a second treatment was ineffective. Pteroylglutamic acid, 25 mgm. daily, was administered orally, following which there was immediate clinical improvement and a gain in weight of 13 pounds in 16 days. In the second article (255) two cases of celiac disease (infantile sprue?) were encountered with "typical clinical features" including flat glucose tolerance curves and megaloblastic bone marrow. Both cases responded to daily dosage with 5 mgm. of pteroylglutamic acid with striking clinical improvement and reticulocyte responses of more than 25 per cent.

Glossitis of pellagra. It was reported (256) that the glossitis of pellagra started to respond within two days to the daily oral administration of 10 mgm. of pteroyl glutamic acid. By 5 days the glossitis had disappeared completely in one case and in a second case there was "evidence of a great deal of healing". The patients had previously shown glossitis which had been successfully treated with niacinamide. These observations may indicate an interesting relationship between niacinamide and pteroylglutamic acid deficiencies in pellagra.

Radiation sickness. Pteroyltriglutamic acid was administered at the rate of 5 mgm. daily for 6 days to 8 patients with leukopenia resulting from local intensive x-ray treatment of carcinoma of the cervix. Elevations of the leukocyte count were noted, but the amounts of pteroyltriglutamic acid available were so limited that the results obtained were not considered to lead to definite conclusions (257). A group of patients with lymphoblastomas received radiation therapy which resulted in an aggravation of anemia and neutropenia. When a crude preparation of pteroylglutamic acid was fed by mouth in addition to the use of radiation therapy in 69 of the patients, it seemed that the deleterious and depressant effects of radiation on the bone marrow were decreased, the frequency of necessary transfusions was lessened, and the period of hospitalization was lessened (258).

Miscellaneous observations. It was noted in India that certain cases of chronic diarrhea responded to treatment with 40 to 60 mgm. of pteroylglutamic acid

daily. Therapy restored the stools to normal or approximately so within two to five days. It was suggested that in long-standing diarrhea a nutritional factor prolonged the production of abnormal stools and that pteroylglutamic acid appeared to correct this defect (259).

The finding that edema occurs in "vitamin-M" deficient monkeys (260) is paralleled by a recent case report of the disappearance of "nutritional edema" in 7 days in a one-year-old child upon treatment with pteroylglutamic acid (261).

TABLE 5

Blood dyscrasias reported not to respond to pteroylglutamic acid

CONDITION	NO. OF CASES	REFERENCE
Anemia of premature infants	18	(248)
Hypochromic macrocytic anemia	5	(248)
Chronic hypoplastic anemia	2	(248)
Mediterranean anemia	1	(248)
Subacute myelogenous anemia	1	(248)
Acute lymphatic anemia	1	(248)
Sickle cell anemia	1	(248)
Hypoplastic marrow	6	(264)
Monocytic leukemia	5	(264)
Leukopenia in virus influenza	3	(264)
Macrocytic anemia secondary to cirrhosis	1	(264)
Anemia associated with portal cirrhosis	4	(227)
Acquired hemolytic anemia	1	(264)
Chronic myelogenous leukemia	1	(264)
Chronic lymphatic leukemia	1	(264)
Aplastic anemia	3	(222)
Iron deficiency anemia	4	(222)
Anemia associated with leukemia	3	(222)
Anemia associated with Weil's disease	1	(227)
Subacute lymphatic leukemia	1	(227)
Refractory anemia following insecticide exposure*	4	(257)
Refractory anemia, other causes*	4	(257)
Leukopenia following x-ray treatment* in Hodgkin's disease	1	(257)
Leukopenia following sulfonamide treatment*	1	(257)

* Treatment with 5 mgm. pteroyltriglutamic acid daily for 6 days.

Relief of two cases of dermatitis was observed in patients who received supplementation with a crude concentrate of pteroylglutamic acid prepared from liver (262). A synthetic pteroyldiglutamic acid was found to produce remission in two relapsed cases of pernicious anemia and one case of nutritional macrocytic anemia (263). One of the cases received 20 mg. daily by mouth for 10 days.

Blood dyscrasias not responding to pteroylglutamic acid. Information regarding these is summarized in table 5.

Discussion. An attempt to define two broad groups of megaloblastic anemias

on the basis of recent results with pteroylglutamic acid is made in table 6. No clear-cut differentiation between the two groups is implied, indeed, it seems that sprue embodies certain characteristics of both groups. A metabolic defect in sprue is indicated by its tendency to relapse and by its response to refined liver extract; in one case report (192) an effect of liver extract on pteroylglutamic acid metabolism in sprue is indicated. A dietary deficiency in sprue is implied by the effects of the accompanying diarrhea and frequently by the nutritional history of the patients.

It has been pointed out that some cases of nutritional macrocytic anemia not only fail to respond to the erythrocyte maturation factor but also do not respond

TABLE 6

Differentiation of two groups of megaloblastic anemias

CHARACTERIZATION	GROUP 1	GROUP 2
Hemopoietic response to:—		
a. Erythrocyte maturation factor of concentrated injectable liver extracts	+	−
b. Lean beef + normal human gastric juice	+	−
c. Pteroylglutamic acid	+	+
Occurrence	May occur on "normal" diet	On deficient diets, usually in tropics
Suggested cause	Metabolic defect	Dietary deficiency of pteroylglutamic acid
Analogue in experimental animals	Unknown	Pteroylglutamic acid deficiency in monkeys, chickens, rats
Examples	Addisonian pernicious anemia	Tropical macrocytic anemia

to feeding lean beef with or without normal human gastric juice, as a source of the extrinsic factor (18). It has been observed that dietary pteroylglutamic acid deficiency in chicks and rats is not alleviated by lean beef (265).

The metabolic disturbance in addisonian pernicious anemia not only involves pteroylglutamic acid but in some patients also includes neurological disturbances which in certain cases may be controlled by liver extract and not by pteroylglutamic acid.

Further light on the classification of the megaloblastic anemias may result from studies in the utilization of pteroylheptaglutamic acid (p. 86). Another possibility for biochemical investigation may lie in additional studies with thymine, which in large doses has been reported to produce remission of pernicious anemia (p. 84) and sprue (p. 90).

Excretion. Only very small amounts of "folic acid" were reported to occur in human urine (266). It was found (50) that the daily urinary excretion of

L. casei factor by 15 normal individuals averaged less than 1 per cent of the probable dietary intake.

Studies of the daily urinary and fecal "folic acid" excretion were measured by assay with *S. fecalis* R (267). Seven young men on a "normal" diet served as experimental subjects. The average daily "folic acid" excretion was about 4 micrograms in the urine and 300 micrograms in the feces. The average daily dietary intake was found to be only 62 micrograms, which seems to be very low, although the diet was not described. In a further communication (268) the effect of supplementation with various vitamins, including 90 micrograms of pteroylglutamic acid daily, was measured in the case of 2 of 7 subjects on a "restricted" diet. Urinary excretion of pteroylglutamic acid was not increased by the supplementation, but the fecal excretion was possibly increased somewhat. Again, the fecal excretion exceeded the intake. The extent to which fecal pteroylglutamic acid is nutritionally available is at present unknown. The occurrence of nutritional macrocytic anemia presumably as a result of dietary pteroylglutamic acid deficiency (4, 21, 22, 23, 24) may indicate that "intestinal synthesis" is ineffective as a source of pteroylglutamic acid under certain conditions, although the production of pteroylglutamic acid deficiency in human subjects on purified diets has not been reported.

It was reported (192) that the daily urinary excretion of pteroylglutamic acid rarely exceeds 5 micrograms and is usually 2 to 4 micrograms on ordinary diets. Oral or parenteral administration of pteroylglutamic acid led within 24 hours to an excretion of 15 to 75 per cent of the amount administered depending on the size of the dose; one individual excreted 16 per cent of one mgm. intramuscular dose, while on a dosage of about 10 mgm. daily either orally or parenterally the excretion usually ranged between 35 and 50 per cent.

Doses of 5 to 16 mgm. of pteroylglutamic acid were fed to 9 normal subjects and 9 hospital patients (269). The normal subjects before receiving pteroylglutamic acid had an average daily urinary excretion of between 2 and 3 micrograms of pteroylglutamic acid. They excreted an average of 28.5 per cent of the administered dose. Most of the excretion took place the second and eighth hours after dosage. The hospital patients excreted much lower percentages of the administered dose. Oral administration of 5 mgm. of sodium pteroylglutamate was found (270) to result in excretion of between 44 and 57 per cent of the administered dose within 6 hours. After 24 hours the excretion fell to basal levels.

Pharmacology. Studies of the pharmacology of pteroylglutamic acid indicated that the substance had a low acute and chronic toxicity and showed an almost complete absence of side reactions even when the dose was far above the therapeutic range (271). Mice, rats, guinea pigs, rabbits, cats and dogs were used in the studies. Renal damage was observed in some of the animals following intravenous injection of large doses of sodium pteroylglutamate. The substance did not affect the respiration or the blood sugar and the effects on the blood pressure and the isolated intestine were of a minor order. In chronic experiments, the daily administration of 5 mgm. per kilo intraperitoneally to rabbits and

rats for two months produced no unfavorable reactions. In a similar period, daily intraperitoneal injections of 50 mgm. per kgm. to rabbits and 75 mgm. per kgm. to rats produced some changes in the tubules of the kidney, but no deaths.

The acute toxicity was low. Rats and mice tolerated approximately 200 mgm. per kgm. intravenously, with no evidence of action.

Relation of pteroylglutamic acid to an anemia produced in dogs. An anemia was reported to occur in dogs which received choline by stomach tube or acetyl choline by injection. Acetyl-choline-like activity was detected in trichloracetic acid filtrates of the serum of blood drawn from dogs one to 1½ hours after the oral administration of 200 mgm. of choline chloride. Trichloracetate was not removed from the filtrate before the acetyl choline assay was made. The activity was diminished in the serum of dogs which had previously received liver extract or pteroylglutamic acid. Cholinesterase activity of the serum was increased by incubation with pteroylglutamic acid or liver extract at 37°C. In two normal human subjects, administration of 5 to 7.5 mgm. of pteroylglutamic acid by mouth led to increases of 33 per cent and 16 per cent in their serum cholinesterase activities within 5 hours. It was concluded that liver extract and pteroylglutamic acid acted by increasing the formation of choline esterase (272). Further experiments indicated that the rate of regeneration of the cholinesterase activity of the serum of diisopropylfluorophosphate treated-dogs was increased by the administration of liver extract or pteroylglutamic acid (273). No confirmation of these results has been reported. No anemia-producing effect of choline was observed in various experiments with human subjects (274, 275, 276).

Pteroyltriglutamic acid, pteroylglutamic acid and mammary tumors in mice. A series of studies was made of the effect of intravenous injections of yeast extract on spontaneous breast tumors in mice (277, 278, 279, 280). Spontaneous tumors were studied in the earlier experiments and later (281) tumors were developed in Rockland mice by transplantation with Sarcoma 180. The mice were then used as test animals. They were kept on a normal diet and the effect of four intravenous injections over a period of 48 hours 8 days after the transplantation was judged by comparing sizes and weights of tumors in matched treated and untreated mice. Inositol alone among crystalline factors of the B complex was reported to inhibit the rate of tumor growth over a range of 50 to 250 micrograms daily when administered by intravenous injection, but subcutaneous or oral administration were ineffective (282). Inhibition of tumor growth in this test was reported to be produced by injecting a concentrate of "folic acid" 0.16 to 5.0 micrograms, or by injecting pteroyltriglutamic acid, 0.0063 to 0.40 microgram (305). Studies with spontaneous breast tumors in mice (283) showed that intravenous injections of pteroyltriglutamic acid, 5 micrograms daily, led to complete regressions of spontaneous breast cancers in mice in the case of 43 per cent of a group of 89 animals. No tumors disappeared among the 60 controls. Mice from three strains were used. In contrast, pteroylglutamic acid had no inhibitory effect and it was reported that primary tumors in mice receiving 100-microgram doses of pteroylglutamic acid intravenously grew more rapidly than the tumors of untreated controls (284).

Further experiments with the transplanted tumors were reported (285). Xanthopterin was found to be inhibitory. It was reported that leucopterin antagonized the inhibitory effect of xanthopterin. Studies of the effects of mutually competitive substances on transplanted tumors were reported (286). Inositol was an inhibitor of tumor growth and was neutralized by p-aminobenzoic

TABLE 7

Media described for the assay of pteroylglutamic acid

INGREDIENT	AMOUNTS PER 10 ml. AND BIBLIOGRAPHIC REFERENCE				
	(8)	(287)	(288)	(98)	(289)
	mgm.	*mgm.*	*mgm.*	*mgm.*	*mgm.*
Na acetate	60	60	60	20	200*
K_2HPO_4				50	
Na citrate					250†
Acid-hydrolyzed casein	50	50	50	50	50
Glucose	100	100	100	100	200
Cystine	1.0	1.0	1.0	1.0	2.0
Tryptophane	1.0	0.5	1.0	3.0	2.0
Adenine		0.1	0.05	0.1	0.1
Guanine		0.1	0.05	0.1	0.1
Uracil		0.1	0.05		0.1
Xanthine		0.1	0.05	0.1	0.1
Asparagine			2.5		1.0
Norited peptone					Present
dl-Alanine					2.0
	μg.	*μg.*	*μg.*	*μg.*	*μg.*
Thiamine		1.0	1.0	2.0	2.0
Riboflavin	1.0	2.0	2.0	2.0	2.0
Nicotinic acid	2.0	1.0	2.0	6.0	6.0
Pyridoxine		1.0	4.0	12.0	12.0
Ca pantothenate	5.0	1.0	2.0	4.0	4.0
Biotin		0.002	0.05	0.004	0.004
p-aminobenzoic acid					0.1
	ml.	*ml.*	*ml.*	*ml.*	*ml.*
Salts A‡	0.05	0.025	0.05		
Salts B§	0.05	0.025	0.05	0.05*	0.05

* For *L. casei*.

† For *S. fecalis* R.

‡ K_2HPO_4, 5 gram; KH_2PO_4, 5 gram; H_2O, 50 ml.

§ $MgSO_4,7H_2O$, 10 gram; NaCl, 0.5 gram; $FeSO_4,7H_2O$, 0.5 gram; $MnSO_4,2H_2O$, 0.337 gram; H_2O, 250 ml.

acid or pyridoxine; desthiobiotin inhibited tumor growth and was neutralized by biotin.

Microbiological assay methods. A variety of methods has been proposed (8, 186, 287, 288, 289, 98) for the microbiological assay of pteroylglutamic acid. A number of attempts to improve the basal media have been made by increasing the amounts of vitamins, amino acids and the supplementary factors, and by increasing the amounts of glucose and buffering capacity to permit a higher maximum growth. These media are described in table 7.

The two organisms which have been used for assay purposes are *S. fecalis* R and *L. casei*. *S. fecalis* has the advantage of giving more reproducible results and of requiring shorter incubation periods than with *L. casei*, but it requires approximately 5 times as much pteroylglutamic acid as *L. casei* to give half maximum growth, thus limiting the assay of very low-potency materials.

Growth with *S. fecalis* R can be measured turbidimetrically after 16 hours or after 40 hours by titrimetric methods. *L. casei* requires an additional growth stimulant to attain maximum growth during the first 16 hours. This growth stimulant, which has been termed "strepogenin" (290) is liberated from casein by tryptic digestion. The acid-hydrolyzed casein usually used as a nitrogen source in microbiological assay media is devoid of strepogenin and thus it is not possible to conduct an assay with *L. casei* in 16 hours for pteroylglutamic acid with such media. A method has been devised (291) to permit a 16-hour assay with *L. casei* by using a tryptic hydrolyzate of casein as a source of amino acids and strepogenin. There have been insufficient published reports on this method, however, to properly assess its value.

In the assay of enzymatically hydrolyzed conjugates *L. casei* will frequently give higher results than those obtained with *S. fecalis*. This is probably due to the fact that *L. casei* will respond to partially hydrolyzed conjugates which are inactive for *S. fecalis* R.

The method of choice would seem to be that of Teply and Elvehjem using either *S. fecalis* or *L. casei*. The medium has a high concentration of buffers and glucose which permits a high maximum growth and contains added quantities of *dl* alanine which has been shown to markedly stimulate growth in the presence of suboptimal amounts of pteroylglutamic acid (292).

The importance of using a pure grade of cotton for plugs is indicated by the observation (293) that nonabsorbent cotton may contain appreciable amounts of pteroylglutamic acid.

Pteroylglutamic acid is very labile at pH 7 to direct or indirect sunlight but four hours exposure to artificial light produced only 10 per cent destruction (294).

The need for appropriate enzymatic hydrolysis of conjugates to precede microbiological assay has already been discussed. In the hydrolysis of natural materials two steps probably occur, first, the release of pteroylglutamic acid or its conjugates from the tissue to give a soluble form and second, the hydrolysis of the soluble but inactive conjugates to microbiologically active compounds. It was found that while taka-diastase cannot hydrolyze all the conjugates present (295, 189) it does aid in releasing the factor from tissues (184). Autoclaving at pH 4 or with 2N potassium hydroxide has been found effective (296) in splitting a conjugate obtained from liver. The use of alkaline hydrolysis has the disadvantage that while pteroylglutamic acid is stable to alkali under anaerobic conditions, it is quickly destroyed by alkali in the presence of oxygen (163). It was also found that (163) pteroyltriglutamic acid could be hydrolyzed anaerobically by alkali but that racemization of the pteroylglutamic acid which was released occurred. A comparison was made of a variety of chemical and enzymic methods for the liberation of pteroylglutamic acid (297). Hydrolysis by auto-

claving at pH 4, by autoclaving with 0.1N potassium hydroxide, and by digestion with taka-diastase and with conjugase from chicken pancreas were compared. With certain tissues e.g. chicken kidney, taka-diastase digestion gave higher results than digestion with conjugase from chicken pancreas. However, with other products, such as yeast extract, conjugase treatment gave the highest values. These data show that there is no digestion method which will yield the highest result with all types of products. The authors (127) have found digestion with taka-diastase, followed by hydrolysis with chicken pancreas conjugase to give higher results than treatment of either enzymic preparation alone.

Assay with chicks. The assay of "vitamin B_c" with chicks on a purified diet was described (80). The chicks were placed on the basal diet until they became anemic, following which the supplements were administered. A prophylactic method has been used more recently (186, 298, 299, 300) based on growth and examination of the blood. The chick assay appears to express the total pteroylglutamic acid content of the sample including both the free and conjugated forms (186).

The use of rats and monkeys for assay animals has been described in detail (301).

Distribution of pteroylglutamic acid. A number of studies have appeared which describe the assay of foods and other natural products for pteroylglutamic acid. The interpretation of some of the early results is complicated by several observations. Pteroylglutamic acid exists naturally to a considerable extent in the form of conjugates which are inactive in the microbiological assay until treated with a specific enzyme preparation which liberates pteroylglutamic acid (185). Even when a preparation of the enzyme is used, there is no criterion for the completion of the hydrolysis, and this consideration is brought into sharp focus by the reported existence of inhibitors of the enzyme system in natural foods (240). Attention has been drawn to the growth promoting effect of thymine as a possible complicating factor in the interpretation of the assay (123). The availability of pteroylglutamic acid conjugates in the nutrition of animals appears to be good (37, 68, 70, 92) although questions have been raised as to the utilization of conjugates by certain species (43) and by pernicious anemia patients (235, 236).

The "folic acid" content of a number of natural foods was carried out by assay with *S. fecalis* R (302). Taka-diastase was used in the preparation of the extracts. This enzyme preparation has been reported not to liberate pteroylglutamic acid completely from its conjugates (36). "Folic acid" showed the greatest losses due to cooking of all the B vitamins studied; cooking losses among meats ranged from 46 per cent in halibut to 95 per cent in pork chops, and in vegetables from 69 per cent in cauliflower to 97 per cent in carrots. The authors postulated that the vitamins may become "bound" in tissues during cooking. Muscle meats ranged from 0.7 to 2.0 micrograms of "folic acid" per gram of fresh tissue, liver 3 to 6 micrograms and vegetables 0.5 to 2 micrograms. The "folic acid" content of samples of various canned foods were assayed for "folic acid" with *S. fecalis* R and *L. casei* following incubation with taka-diastase for 24 hours at 37° (303). Considerable disparity between *S. fecalis* and *L. casei* values was

encountered, for example yellow corn was found to contain 0.017 microgram per gram by *S. fecalis* R and 0.056 by *L. casei*. Most of the values obtained with canned foods were quite low, averaging less than 0.1 micrograms per gram, except for canned spinach.

Similar assay methods were applied to a study of meats (304). Muscle meats were found to contain 0.06 to 0.33 microgram per gram of fresh tissue, liver 0.4 to 1.5 micrograms and kidney 0.3 to 0.6 microgram. Cooking resulted in destruction ranging up to 92 per cent of the original content.

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CONDITIONING FACTORS IN NUTRITIONAL DISEASE[1]

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MALNUTRITION MAY BE CAUSED by a number of factors other than inadequate dietary intake. These include conditions that interfere with the absorption or utilization of nutrients, or those that increase their requirement, destruction or excretion. These "conditioning factors", as Jolliffe terms them (237), have one point in common: they increase body requirements for specific nutrients or precipitate nutritional deficiencies on diets that would otherwise be adequate were such factors not operative. The effects of "conditioning factors" on vitamin deficiencies have been the subject of earlier reviews (236, 237, 239, 391); it is the object of the present contribution to summarize their effects on other nutrients as well. The present review makes no attempt to be a complete compendium of the literature but is designed as a critical survey of pertinent material in a few main sections of the field.

Factors Interfering with Absorption. A. *Fat soluble Vitamins.* Fat is necessary for the efficient absorption of carotene and vitamin A (26). Wilson (441) found that in men on a fat-free diet the absorption of carotene was nearly halved; while Ahmad (7) observed that in animals on a fat-free diet only ten to twenty per cent of carotene was absorbed when dissolved in ethyl laurate with the percentage rising to eighty or ninety per cent when ten per cent fat was also taken. The importance of fat for the absorption of vitamin A is largely academic, for vitamin A, particularly in foods, is almost invariably found associated with fat; the small amounts of fat in highly concentrated medical preparations of vitamin A may, however, decrease their value. Rough clinical proof that fat, in normal amounts at least, aids absorption is given by the large number of investigations which show that in all diseases where the absorption of fat is impaired, absorption of vitamin A and vitamin A-active carotenoids is also impaired. These include a number of conditions consisting of celiac disease (45, 65, 211, 292), sprue (6), fibrosis and cystic disease of the pancreas (15, 146, 292), congenital atresia of the bile ducts (14, 292), intestinal obstruction, ulcerative colitis, dysentery and diarrhea. Fever also exerts an adverse effect on the absorption of vitamin A and A-active carotenoids (211, 282), an effect most probably due to impaired digestion of fats. Among twenty-nine patients with pulmonary tuberculosis, most of them exhibiting symptoms of intestinal tuberculosis as well, the absorption of vitamin A was

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found to be less efficient than in healthy individuals (47). Absorption of vitamin A was similarly impaired in persons with lambliasis or giardiasis (247).

Evidence is available indicating that carotene requires for its absorption the presence of bile in the intestinal tract. In those conditions in which bile is excluded completely or partially from the intestinal tract or whenever there is a deficiency of bile salts, bile salts must be given as a supplement in order to assure proper absorption (162). Vitamin A appears to be absorbed, however, in the absence of bile or bile salts, though absorption of it is more efficient when one or the other is present (46, 47, 162, 163, 247, 388).

Liquid paraffin seriously interferes with the absorption of carotene by dissolving it from the food in the intestine, with the result that it is excreted with the paraffin in the feces (91, 348). Curtis and Ballmer (90) have shown by careful work on man that on a high carotene diet the carotene level in the blood only rises to one-half the normal value when 20 cc. of paraffin are taken thrice daily after meals; taking it twice daily has nearly as bad an effect, and even taking it once at night has some effect. Similar results have been obtained in work on animals (116, 227). Available evidence indicates that liquid paraffin does not materially interfere with the absorption of vitamin A (16, 116).

In general, the absorption of vitamins D, E and K is believed to be influenced by the same factors that affect vitamin A. Taylor and co-workers (410) found that dogs with biliary fistulae did not absorb vitamin D when given by mouth unless bile salts were given at the same time. Other investigators, among whom is Heymans (212) have confirmed these results by very similar experiments on rats or dogs. Brinkhous and Warner (50) report that dogs with chronic biliary fistulae develop within seven to nine months both the nutritional muscular dystrophy and testicular degeneration typical of a deficiency of vitamin E. In one dog, the muscular dystrophy was arrested and improved when bile was fed by mouth. Greaves and Schmidt (164) have obtained similar results in rats. Available evidence indicates that the bile salts are also essential for the proper absorption of natural vitamin K. Greaves and Schmidt (165) made the important observation that in bile fistula rats the clotting time was markedly increased when the prothrombin level of the blood dropped to a level of 20–30 per cent of the normal. This level of prothrombin was raised by feeding 2–3 cc. of beef bile daily or massive doses of vitamin K. The best results were obtained by combining bile and vitamin K. Similar results have been observed by Smith et al. (380) in dogs, and Brinkhous and co-workers (49) in man. Synthetic vitamin K analogues, however, particularly the water-soluble ones, do not need bile salts for their absorption if given in adequate amounts (246).

Available data indicate that liquid paraffin, or mineral oil, hinders the absorption of vitamins D, E and K. Smith and Spector (381) observed that rats on a non-rachitic diet developed rickets if as much liquid paraffin was added to their food as would correspond to the amount taken by man for constipation. Five times the normal requirements of cod liver oil were required to counter-balance the effect of this amount of liquid paraffin. Similar results were obtained for vitamins E and K (10, 25, 41, 122, 228).

Although data are lacking for hypovitaminosis D and E, hypoprothrombinemia, the chief manifestation of hypovitaminosis K, has been observed for a number of gastro-intestinal diseases and subsequent to short-circuiting surgical procedures. Vitamin K deficiency may result from excessive vomiting and loss of succus entericus from a drainage tube. In conditions associated with chronic and severe diarrhea, food is hurried through the intestine, so that absorption of vitamin K, and of other vitamins for that matter, is impaired (69). Hypoprothrombinemia has been observed in cases of ulcerative colitis (35, 326), regional enteritis, intestinal obstruction, Banti's disease, gastrocolic fistula, enteroanastomosis, ileac enterostomy, intestinal neoplasm, polyposis coli, and those diseases in which fat absorption is particularly impaired, such as tropical sprue, idiopathic steatorrhoea, celiac disease, and chronic diarrheas in general.

B. *Water soluble vitamins.* Absorption of water soluble vitamins may be impaired by factors resulting in anatomic, chemical or physiologic changes in the gastro-intestinal tract (30). These changes are mediated by reduced absorbing surfaces, altered secretions and hypermotility, and further modified by various alkalis, adsorbents and lubricants introduced by mouth. Vitamin B_1 may escape absorption from the intestine in certain digestive disturbances such as vomiting, diarrhea (100), alterations of the gastro-intestinal mucosa (ulcerative colitis) (240), and defective secretion due to atrophy, inflammation, and neoplastic disease. Operations designed to short circuit the intestines (422), internal and external fistulae and strictures will also lead to diminished absorption (20, 436). Available evidence indicates achlorhydria may impair the absorption of vitamin B_1 (143, 297). Absorption may also be impaired by various adsorbents such as aluminum hydroxide and kaolin.

In general, the factors referred to above apply to other water soluble vitamins as well. Diarrhea has been a causative factor in the production of riboflavin deficiency (31, 68, 364), pellagra (11, 31, 64, 68, 389, 436), and scurvy (3, 451). Frequent reports have appeared on the development of pellagra secondary to lesions such as carcinoma, duodenal ulcer and a malfunctioning gastro-enteric anastomosis which obstruct the stomach and duodenum, and similar reports are available for internal and external fistulae (436).

It has long been known that patients suffering from gastro-intestinal diseases excrete subnormal amounts of vitamin C in their urine. This has been attributed in the past to a low intake resulting from a restricted diet, but the work of a number of investigators suggests that defective absorption may be a more important factor in some cases. Under abnormal conditions of the gastro-intestinal tract associated with abnormal bowel motility (catharsis, diarrhea, ulcerative colitis) marked losses of vitamin C taken by mouth may occur. Thus, these conditions are frequently severe enough to decrease the blood level of vitamin C as well as the excretion in the urine, even when large amounts of the vitamin are given by mouth (301). Available evidence indicates that absorption of vitamin C may be impaired in achlorhydria (5, 451).

C. *Minerals.* Absorption of calcium is governed chiefly by three factors: (1) Vitamin D, (2) the hydrogen-ion concentration within the intestine and (3)

other substances in the diet. The exact mode of action of vitamin D is not known. However, although its chief effect may be exerted in the intermediary metabolism of phosphorus and calcium and in their disposition in the bones, there is little doubt that this factor, in physiologic dosage, plays an important rôle in favoring absorption of calcium from the intestine. Factors interfering with the absorption of vitamin D may thus indirectly contribute to an impaired absorption of calcium.

Under conditions of normal gastric acidity, compounds of calcium with weak organic acids are converted into the chloride and, if retained in the stomach for a sufficient period, even the less soluble basic phosphate may go into solution. The acidity of the duodenum is of considerable importance for the adequate absorption of calcium. Normally, it ranges from pH 2.3 to 7.0; and this factor largely determines whether calcium is primarily in the form of the acid or the basic phosphate. Since the acid phosphate is the more soluble, a high acidity tends to facilitate absorption of calcium. Calcium chloride and acid phosphate are probably absorbed from the duodenum before the gastric acidity is neutralized and, subsequently, continued absorption may be favored by the formation of organic acids (carbonic and lactic) (62, 363). Factors which tend to decrease the acidity of the duodenum will tend to impair absorption of calcium. Achlorhydria and the administration of alkalinizing agents are of particular importance in this regard.

Calcium absorption may be impaired by a number of substances commonly ingested in our diet. The oxalate content of our foods is a case in point. Spinach, New Zealand spinach, Swiss chard, beet tops, lambs quarters, poke, purslane and rhubarb leaves contain, on a dry weight basis, as much as 10 per cent oxalic acid (for the most part in the form of its salts). Traces of oxalic acid are also found in many other fruits and vegatables (257). Fincke and Sherman (144) showed that when rats were fed diets containing 0.33 per cent calcium, half of which was supplied by milk and half by spinach, only 40 per cent of this calcium was deposited in the bones of the growing animals, whereas when the spinach was replaced by kale (an oxalate-free leafy green), the deposited calcium rose to 80 per cent. Kohman (258) later reported similar findings and showed further that a diet which was satisfactory in calcium for growth could lead to injury by the addition of oxalate-containing leafy greens. However, if enough calcium were added to the defective diets they again became innocuous. There seems to be no doubt that oxalates do interfere with efficient calcium utilization. Whether this interference leads to a deficiency depends upon the oxalate and calcium contents of the diet.

Phytic acid is another substance commonly present in our diet whose ingestion may interfere with the absorption of calcium. Some twenty years ago, Mellanby (293) made the important observation that the addition of certain cereals to diets low in calcium led to severe rickets in growing animals. Subsequent work (52, 201, 221, 294, 412) has demonstrated that inositol-hexaphosphoric acid (phytic acid) was the substance present in cereals responsible for this rachitogenic effect. Sodium phytate is similarly rachitogenic, although phytin (the Ca-Mg

salt) is without effect. The rachitogenic effects of phytic acid or its sodium salt are counteracted by calcium. Apparently, phytic acid interferes with the absorption of calcium, either by precipitation or by converting it into a non-ionized form which is not absorbed from the intestine. As in the case of oxalates, whether this interference leads to a deficiency depends upon the phytic acid and calcium content of the diet.

Normal nutrition depends more upon an adequate intake of both calcium and phosphorus than upon the calcium/phosphorus ratio; the latter, however, acquires more significance as the absolute values for either element approach the minimum requirement levels. An excessively high phosphorus intake tends to inhibit absorption of calcium, especially if the latter is supplied in insufficent amounts. A similar inhibiting effect is apparently exerted by an excess of Mg and of K. Diets unusually high in fat may tend to inhibit absorption of calcium from the intestine, due to the formation of insoluble calcium soaps. A similar situation has been observed in celiac diseases, sprue and so-called "idiopathic" steatorrhea due to impaired intestinal absorption of fats and the resulting formation of relatively insoluble calcium soaps. Inadequate absorption may also result from protracted diarrhea, due to the rapid passage of intestinal contents through the bowel (201).

Absortion of phosphorus is diminished by factors which favor the formation of poorly soluble salts of phosphoric acid. This can be demonstrated strikingly by experimental administration of beryllium which, through the formation of insoluble beryllium phosphate, prevents absorption of phosphoric acid and results in a characteristic form of rickets (180). Similar inhibition is produced, to a much smaller degree, by an excess of other cations, such as calcium, strontium, manganese, magnesium, barium, aluminum and thallium. Waltner (429) showed that rickets could be produced in rats by the addition of soluble iron salts to a non-rachitogenic diet, a finding confirmed by Brock and Diamond (51). Severe phosphorus depletion was produced in young chicks by Deobald and Elvehjem (104) who treated their normal ration with soluble iron and aluminum salts equivalent to 0.5 and 0.75 per cent of the theoretical amount required to combine with the dietary phosphorus as $FePO_4$ and $AlPO_4$. Normal bone ash values of 50 to 60 per cent were reduced to 25 per cent. The latter investigators warned that the clinical usage of very high doses of iron in the treatment of anemia might seriously interfere with phosphorus utilization.

A number of factors may contribute to an impaired absorption of iron (186). The relatively high pH in the duodenum facilitates the formation of insoluble basic iron compounds. The alkalinity of pancreatic juice and the relative insolubility of iron salts of bile acids probably interfere with the absorption of iron. Absorption appears to be hampered by the absence of free HCl and of bile and is also influenced unfavorably by administration of alkalis. It has been shown by Brock and Diamond (51) and others that phosphates have a deleterious effect on the absorption of iron, probably because of the precipitation of ferric phosphate. Absorption of iron is probably decreased by diarrhea and inflammation of the gastro-intestinal tract, but conclusive evidence is lacking. Absorption

may also be decreased by phytic acid (278). Hahn, Bale and Whipple (187) have recently reported still another factor that may seriously interfere with iron absorption. These investigators demonstrated that inflammation following the injection of turpentine in the subcutaneous tissue of dogs markedly reduced the absorption of iron from the gastro-intestinal tract. Subsequent to abscess healing, the rate of absorption returned to normal.

D. *Miscellaneous.* Carbohydrate, protein and fat are present in the diet largely as complex foodstuffs which must be broken down to their respective monosaccharides, amino acids, and fatty acids before adequate absorption may occur. Factors interfering with digestion will tend, therefore, to impair absorption. The diarrheal diseases are a case in point. In colitis, dysentery, intestinal parasitism, intestinal tuberculosis, sprue and pellagra, food is rushed through the intestinal tract so rapidly that little time is left for digestion, solution and absorption. The digestive secretions may be altered in amount and kind. In theory, this might be expected to be a serious cause of malnutrition; in practice, however, such is not the case. In spite of the high specificity of the digestive enzymes, the body is not entirely dependent on any single source for digestion. Thus, such radical measures as complete gastrectomy, removal of the duodenum, removal of the pancreas or of parts of the small intestine do not seriously interfere with the degree of digestion. On feeding large amounts of certain foods, some derangement of digestion may be demonstrated under the foregoing circumstances, but the degree of digestion that does occur is impressive. A more serious cause of inadequate absorption would be an impairment of the absorbing surface of the gut, present in a number of conditions, including chronic ulcerative colitis, sprue, and possibly food allergy.

Digestion and absorption of protein may be impaired by a number of factors in addition to those listed above. Recent work indicates that the pH 4.2-soluble, acetone-insoluble fraction of raw soybeans has a growth-retarding effect on both chicks (191) and rats (256). Inasmuch as Ham and Sandstedt (190) and Bowman (42) demonstrated the presence of a trypsin inhibitor in the pH 4.2-soluble fraction of unheated soybean flakes, the question of the identity or non-identity of the trypsin inhibitor with the growth inhibitor reported above is very much to the fore. Since both are present in the acid-soluble fraction of raw soybean protein, the two may be identical. Evidence is available that the digestibility of milk proteins may be decreased by the administration of cocoa (273, 314).

Normal absorption of carbohydrate does not occur if there is an anterior pituitary deficiency. This probably depends for the most part upon the secondary hypofunction of the thyroid gland, for the same result may be obtained after removal of the thyroid gland when the hypophysis is intact. Furthermore, the defect in absorption accompanying hypopituitarism may be relieved by the administration of thyroid extract (351). The adrenal cortex influences carbohydrate absorption through its regulation of the sodium chloride exchange in the body. The absorption of carbohydrate from the intestine is subnormal in adrenal cortical deficiency, but can be restored to normal without the use of adrenal cortical extracts if the sodium chloride of the blood is raised to normal levels by adequate salt intake (13).

Factors Causing Destruction or Inactivation of Vitamins. Several of the water soluble vitamins are readily destroyed or inactivated in the gastrointestinal tract prior to absorption. Melnick, Robinson and Field (297) have demonstrated that thiamine was stable in gastric juice over a pH range of 1.5 to 8.0 but in a more alkaline pH the vitamin was readily destroyed. When antacids were added, destruction of thiamine was complete. In patients with achlorhydria significant destruction of thiamine may occur in the gastric juice (267) or further along the gastrointestinal tract where the vitamin comes in contact with the bile or pancreatic juices (143). Melnick and co-workers (296) have shown that when an oral dose of vitamin B_1 is taken by a normal subject, a much smaller fraction is excreted than when the same dose is ingested during or just after a meal. The difference may be due to the instability of the vitamin in a more alkaline gastrointestinal tract when gastric secretion is at a minimum. Alt, Chinn and Farmer (12) obtained a 65 per cent destruction of ascorbic acid in three hours at a pH of 7.95, representing achlorhydric gastric juice. Vitamin E may be destroyed during digestion in the presence of rancid fats (284, 285, 291). Destruction of carotene has similarly been reported (374).

Certain foodstuffs contain substances that may destroy or inactivate B vitamins. A number of workers have demonstrated that carp, pike, smelt and other raw fish when mixed in the diet of foxes precipitate a condition known as Chastek paralysis (168, 169, 217, 320). The animals develop anorexia, followed in a few days by weakness, hyperesthesia, ataxia and death. At autopsy, bilaterally symmetrical lesions are found in the grey matter of the brain, mainly in the paraventricular region and similar, pathologically, to the cerebral lesions of Wernicke's hemorrhagic polio-encephalitis of man (8, 136). The condition is preventable or curable by administration of thiamine (169) and is identical to that observed in adult silver foxes fed a diet deficient in vitamin B_1 (217). Subsequent work has demonstrated that Chastek paralysis is due to a thiamine deficiency resulting from the destruction of this vitamin by a heat labile enzyme-like substance (subsequently termed "thiaminase", (295), present mainly in the viscera, head, skin and scales of certain fish (38, 105, 169, 259, 295, 366, 367, 390). Thiamine deficiency has been observed in cats fed an exclusive diet of raw carp or herring (377); and Melnick et al. (295) report a 50 per cent destruction of dietary thiamine in persons fed diets containing raw clam. Putney (336) had recently reported the presence of an anti-thiamine factor in raw meat. Still another thiamine-destroying factor has been reported by Bhagvat and Devi in certain cereals, legumes and oil seeds (39). The properties of the factor indicate that it is not an enzyme and therefore not identical to the substance responsible for Chastek paralysis. When 50 micrograms of thiamine were added to 50 cc. of a buffered (pH 5.6) 10 per cent suspension of rice polishings, ragi, wheat germ, cottonseed, linseed, and certain other seeds, as much as 90 per cent of the thiamine was destroyed overnight. In certain cases, after only seven minutes of treatment, as much as 75 per cent of added thiamine had disappeared. Thiamine deficiency may also be induced by the feeding of live yeast (318). In the latter instance, however, the effect is believed due to the withholding of the vitamin from absorption rather than direct destruction.

Certain strains of bacteria appear to be able to decompose vitamin C in vitro, but whether they exert this effect in the intestine is open to question. Stepp (394) and Einhauser (121) have shown that strains of *E. coli* and *B. paratyphosus* were capable of destroying vitamin C. Kendall and Chinn (249) found that certain bacteria, isolated from the stomach and intestinal contents, were able to ferment the vitamin; and Young et al. (455, 456) demonstrated that numerous strains of enteric organisms, including *E. coli*, *Salmonella*, *Eberthella*, *intestinal streptococci*, *vibrios*, and *Proteus morgagni* could decompose vitamin C. While normally this may be of no significance, in some pathological conditions it might account for a clinical deficiency of vitamin C, even in cases where intake is adequate. Thus cases of clinical scurvy have been reported which failed to respond to oral ascorbic acid therapy, although recovery was rapid following parenteral administration.

Biotin deficiency is another condition that may be induced by the ingestion of certain foods. Raw egg white contains a substance capable of combining stoichiometrically with biotin and rendering it unavailable to the organism, presumably by preventing its absorption from the gastro-intestinal tract (183). The isolation and properties of this material, a protein subsequently termed avidin, has been described (448), and its crystallization and analysis effected (330). Avidin is denatured by heat and its ability to bind biotin lost. This explains why raw egg white inactivates biotin physiologically, but cooked egg white does not. Curiously enough, the avidin-biotin complex cannot be broken down by the gastro-intestinal enzymes, yet it can be utilized if injected (182). There is some evidence that biotin can also be inactivated by rancid fats (328).

Factors Interfering with Utilization. As pointed out by Kruse (260) malnutrition denotes a deficiency of essential nutrients in the tissue cells rather than in the diet. Any factor interfering, therefore, with the utilization of an essential nutrient may precipitate a nutritional deficiency despite the composition of the diet or the adequacy of digestion or absorption. The subject has been extensively reviewed by Jolliffe (237) and more recently by Hickman and Harris (213). It is obvious that before a nutrient can be employed by the body it must be transported from the intestinal wall subsequent to absorption to the functional body sites. This journey requires a carrier. For the fat-soluble vitamins, however, the carrier must be a specialized adjunct of the vitamin and necessary for its proper distribution— for instance, a suspended lipide or a phospholipide or protein complex. In the absence of sufficient lipide carrier according to Hickman and Harris, the *efficiency of conveyance* may be depressed (213). Failure of utilization may also result from an interference with the *mechanism of acceptance* (213). Certain tissues have an affinity for specific nutrients and can *accept* or concentrate them from blood or lymph prior to use. The concentration of iodine by the thyroid gland is a case in point (194, 210). Further examples may include the concentration of vitamin C by the adrenals (453), of vitamin A by the liver (426), of vitamin E by heart muscle and mammary gland (290) or of riboflavin by liver and kidney (424). If the mechanism of acceptance is faulty, the efficiency of utilization will be impaired.

Failure of utilization may also result from inadequate conversion of essential nutrients into active physiological components, in which form they participate in body metabolism. Thus carotene must be converted into vitamin A, thiamine to cocarboxylase, riboflavin to flavoprotein, and nicotinic acid to coenzymes I or II. Since the liver is considered to be the principal organ in which these conversions occur, hepatic dysfunction due to cirrhosis or other conditions may manifest itself as a nutritional deficiency of the tissue cell. The high frequency of nutritional disease in alcoholics may be due in part to hepatic dysfunction in such cases. A tissue deficiency of vitamin A has been reported in liver cirrhosis (188, 337, 338), diabetes mellitus (44) and obstructive jaundice (396), presumably due to impaired conversion of carotene to vitamin A; and similar effects have been observed in hypothyroidism (263, 442). According to Wendt (430), thyroid is necessary for the conversion of carotene to vitamin A and for the storage of vitamin A in the liver, a finding in agreement with the observations of Wohl and Feldman (442). Deficiency of protein is another factor that may contribute to poor utilization of nutrients. Trufanov (420) found that synthesis of flavin-adenine-dinucleotide failed to occur in the liver and tissues of rats fed for 50 days on a low-protein diet, although when an adequate diet was subsequently fed, a marked synthesis of dinucleotide took place both in liver and muscle tissue. Therapy with sulphonamides and other drugs may also interfere with utilization of B vitamins. West (432) has shown that sulphapyridine inhibits the activity of nicotinic acid in the dog, although similar results were not obtained in man (237). Evidence is available indicating that radiation sickness may be due to failure of coenzyme formation from thiamine (32, 226, 288, 428) or nicotinic acid (32).

A number of workers have demonstrated that calcium utilization may be adversely affected by the ingestion of large amounts of magnesium. As far back as 1905 Malcolm observed an increased calcium excretion in dogs fed magnesium chloride. Eight years later, Hart and Steenbock found that magnesium chloride and magnesium sulfate fed to swine caused an increased excretion of urinary but not fecal calcium. Soluble phosphates fed with the magnesium, however, decreased the loss of urinary calcium with scarcely any increase in the fecal fraction. Similar results were found in cattle by Palmer et al. (327). Cunningham (89) observed a reduction in bone calcium of rats fed magnesium as the sulfate or carbonate salt, a finding in agreement with that of Buckner et al. in chicks. These findings did not occur when magnesium phosphate was the salt administered (55). Evidence is available indicating that the utilization of calcium as well as phosphorus and protein may be impaired by the ingestion of certain brands of cocoa (309, 314).

Administration of inhibitory structural analogues of the various vitamins constitutes another factor that may impair the utilization of nutrients with a resultant deficiency in the tissue cell. This deficiency may occur in spite of the level of vitamins fed provided, of course, sufficient amounts of analogue are administered. The subject has been extensively reviewed by Roblin (346) and Woolley (445). The most popular hypothesis to explain why analogues exhibit

competitive antagonism with an essential metabolite is that the two compounds vie for space on a portion of an enzyme normally engaged with the metabolite. If the concentration of the inhibitor is great enough in comparison to that of the metabolite, the latter is displaced, and the biological system is thereby deprived of it. The net result is the precipitation of a deficiency characteristic of the structurally related metabolite and reversible by the administration of adequate amounts of the appropriate vitamin. To date, inhibitory structural analogues have been synthesized for thiamine (387, 450), riboflavin (125, 126, 262, 443), nicotinic acid (444, 449), pantothenic acid (261, 281, 383, 384, 446), biotin (106, 128, 272), p-aminobenzoic acid (206, 346), ascorbic acid (447) and vitamin K (298, 325).

Factors that Increase Excretion. A number of factors such as polyuria, lactation, excessive perspiration or administration of therapeutic agents may contribute toward malnutrition by increasing the elimination of essential nutrients from the body. Although such factors are unlikely to be the primary cause of tissue deficiency, they may nevertheless accentuate existing deficiencies or speed up the appearance of symptoms on an inadequate diet. Cowgill et al. (81, 83) have shown that, on a restricted thiamine intake, symptoms of deficiency appeared earlier in dogs receiving a forced fluid intake than in animals permitted to drink *ad libitum*. The forcing of fluids for prolonged periods of time in certain urinary tract infections or the loss of fluids by diuresis in anasarca may have a similar effect in man. Polyuria as present in diabetes mellitus or diabetes insipidus may also result in increased excretion of thiamine and other water soluble nutrients (237). Lactation may seriously deplete body stores of essential nutrients. Loss of calcium is of particular importance in this regard, (225, 286) although a significant loss of B vitamins (137, 398), ascorbic acid (28, 150) and protein (283) also occurs. Data are available indicating that excessive perspiration may result in appreciable losses of thiamine from the body, particularly under tropical conditions or in subjects doing heavy manual work in a hot environment (78). Other investigators, however, have found that the thiamine, riboflavin, pantothenic acid, nicotinic acid and ascorbic acid content of sweat was virtually negligible (197, 233, 302, 360, 413, 452). A significant excretion of sodium chloride may occur as a result of excessive perspiration (406).

Various drugs commonly employed as therapeutic agents may increase the excretion of vitamin C. Salicylates, atropine, aspirin, cincophen, barbiturates, amidopyrine, antipyrine, adrenaline, chloroform, chloretone, paraldehyde, the commoner anesthetics, stilbestrol, estradiol, and sulfonamides all increase excretion of vitamin C (37, 138, 218, 242, 248, 275, 303, 304, 341, 353, 370, 402). Certain foodstuffs have a similar effect. Basu and Ray (27) report that ingestion of cabbage extract resulted in a vitamin C excretion temporarily greater than the intake; and Musulin et al. (317) found that oats and certain fractions of halibut liver oil exerted a similar effect. According to Selkurt et al. (370) the increased excretion of vitamin C, at least that following administration of estrogens, was due to changes in renal tubular absorption. Increased urinary excretion of other vitamins has also been reported. The excretion of thiamine is consider-

ably increased by the administration of mercurial diuretics such as mercurophylline (439), an increase that is not due entirely to increased diuresis, since the concentration of thiamine in the urine is several times greater than normal. Over short periods salicylates may also increase urinary thiamine excretion, although output is subsequently reduced (71). Sure (399) has reported an increased urinary excretion of riboflavin in the thiamine deficient rat. An increase of urinary riboflavin has also been observed in experimental hyperthyroidism (400). Significant losses of vitamin A may also occur. Vitamin A is never excreted by the kidneys of man in good health, except possibly as a breakdown product (415), but it may appear in the urine during illness, being found frequently and in the highest concentrations in pneumonia. A daily output of 3,200 I.U. has been recorded, which ceased abruptly with the crisis (265). In chronic nephritis vitamin A is common in the urine, though in smaller amounts than in pneumonia. Still smaller amounts have been reported in chronic infections, rheumatic fever, skin diseases, pernicious anemia, asthma, cancer and normal pregnancy (265). The effects of magnesium salts on calcium excretion have already been reviewed. A significant loss of calcium may occur in hyperthyroidism and hyperparathyroidism. An increase in the urinary excretion of calcium and phosphorus has been reported following fracture (222).

Excessive loss of protein may occur in a variety of conditions: for example, after hemorrhage (56, 127, 311), operations and injuries (53, 93, 94, 223, 224, 315), gastro-intestinal obstruction (73, 184, 282) or burns (74, 87, 408, 409). Diseases characterized by chronic bleeding (ulcers, intestinal carcinomas, uterine fibroids, etc.) may precipitate hypoproteinemia by depletion of plasma proteins through prolonged blood loss. Excessive protein loss through proteinuria resulting from kidney disease (139, 250, 268, 332) or other causes (63) exacts a severe toll upon plasma proteins and may deplete body protein reserves. Injury to blood vessels, through mechanical or chemical means, or as a result of severe burns, may so alter cell permeability as to permit loss of blood proteins through exudation (79, 216, 276). In the same manner continuous drainage of pus (as in peritonitis, osteomyelitis, etc.) represents an additional pathway for the loss of protein material (333). Damage resulting from surgery may cause not only alterations in cell permeability, permitting leakage of proteins (80), but actual breakdown of tissue protein, as well as severe hemorrhage, thereby contributing significantly to protein loss. Loss of nitrogen resulting from breakdown of body protein is particularly marked in patients with fractures (93, 94, 223, 224), meningococcus meningitis (178), or during the febrile stage of most acute infections. Interestingly enough, in such cases negative nitrogen balances are not appreciably modified by increasing dietary protein nor by supplementing diets with intravenous injections of protein hydrolysates (333). Amino acids may be lost to the body in the form of proteoses or closely related substances which are frequently found in the urine in certain febrile disorders, particularly pneumonia, diphtheria and pulmonary tuberculosis, as well as in peptic ulcer, carcinoma and osteomalacia (63). An increase in the amino acid content of the urine may occur in conditions in which hepatic function is impaired or those characterized by extensive tissue

autolysis. High figures are obtained in acute yellow atrophy of the liver, eclampsia, and in chloroform, phosphorus, cincophen, arsenic and carbon tetrachloride poisoning. Under such circumstances, leucine, tyrosine, glycine, arginine, phenylalanine and other amino acids may appear in the urine in large quantities. Urinary amino acids are also increased in conditions associated with excessive tissue wasting as in protracted fevers (typhoid) (63). In addition to the general loss of amino acids reported above, specific amino acids, or products of their metabolism, may appear in the urine in certain pathologic conditions. Tyrosine, for example, may be excreted in the urine in amounts of 0.9 to 2.0 grams daily in cases of acute hepatic necrosis (271). Tyrosinuria may occur in the presence of extrahepatic foci of autolysis, as in degenerating lung tumors or extensive sloughing of the skin. To a lesser extent it may occur in subacute atrophy of the liver, toxic hepatitis, and, on occasion, in prolonged calculous obstructive jaundice (63). Tyrosinuria may occur as an "inborn error of metabolism" (151). Other "inborn errors of metabolism" include cystinuria, in which 0.4 to 1.0 gram daily of cystine may be lost in the urine and alkaptonuria, characterized by the urinary excretion of homogentisic acid, due to an abnormality in the intermediary metabolism of phenylalanine and tyrosine. Homogentisic acid has also been found in the urine of normal subjects given tyrosine and a diet deficient in vitamin C, disappearing after administration of the vitamin. The latter, however, has no effect upon clinical alkaptonuria (365, 368). Phenylpyruvic acid has been found in the urine of certain patients with mental deficiency and manifestations of extrapyramidal system disturbance (147, 229). The condition is apparently due to impairment of the metabolism of phenylalanine. P-hydroxyphenyllactic acid and p-hydroxyphenylpyruvic acids have been found in the urine of premature infants fed diets of vitamin C-free cow's milk containing 5 grams or more of protein per kilogram. These substances disappeared after administration of vitamin C (269). Histidinuria has been observed both in males and females, in health and disease (421). The condition is particularly pronounced during pregnancy, where it appears about the fifth week of gestation, coincident with the appearance of urinary prolan, and disappears rapidly following parturition (244, 245, 425).

Factors Increasing Body Requirements (Stress Factors). Body requirements for essential nutrients may be increased by physical exertion, fever, drugs, toxins, abnormal environmental conditions, pregnancy, lactation, hyperthyroidism, and related conditions. These constitute what are referred to as "stress factors" in the present review and may be defined as any condition resulting in an increased metabolic requirement for an essential nutrient on the part of a tissue cell. Such nutrients may be employed for purposes of detoxification or they may be used in the increased metabolism of specific tissues. The net result, however, is that nutritional requirements are increased beyond the usual or average range.

A. *Increased metabolism.* Evidence is available indicating that the requirement for many nutritive essentials is proportional to the metabolic rate. Accordingly, body requirements may be elevated in conditions with an increased B.M.R. such as hyperthyroidism, toxic adenomata and malignancy of the thyroid gland,

diabetes insipidus, fever, cardiorenal disease with dyspnea, severe anemias, diabetic pseudodwarfism, leukemia and polycythemia. Considerable work has been reported on the increased requirements of pyrexia, particularly for vitamin C. Clinically diminished urinary excretion and reduction in the blood level of vitamin C occurs in a number of infections, including pneumonia (339), diphtheria (264, 323), osteomyelitis (1), rheumatic fever (2, 345), rheumatoid arthritis (189, 343, 344), tuberculosis (230, 403) and others (140, 141, 198). Faulkner and Taylor (140, 141) found that whereas the average vitamin C content of the blood serum of healthy persons averaged 1.31 mgm. per 100 cc.; patients with infectious diseases had values as a rule fifty per cent lower or about 0.65 mgm. per 100 cc. Examination of the vitamin C reserves of the body (blood, urine, saliva, cerebrospinal fluid) revealed that in pulmonary tuberculosis, pneumonia, diphtheria, and febrile diseases generally the concentration of vitamin C was low as compared with normal standards (22, 23, 57, 58, 203, 205). Similarly, a comparison of the vitamin C content of the organs of experimental animals suffering from infections such as diphtheria and tuberculosis with those of healthy controls revealed that vitamin C stores were significantly reduced in the infected animals (156, 198, 417). The question naturally arises as to whether the increased utilization of vitamin C in febrile diseases is due to elevation of body temperature or to the infectious process *per se*. Reductions in the serum level of vitamin C in artificial pyrexia suggest that elevation of body temperature was the responsible agent (19, 102, 107, 204, 457), presumably by increasing metabolism (fever increases basal metabolism by approximately 7.2 per cent for each degree F.) (112). Osborne and Farmer (324), however, failed to confirm these findings. They found no significant change in the vitamin C blood level before or during pyrexia in patients submitting to temperatures of 102 degrees F. in fever cabinets. Abt and co-workers (4) also concluded from studies on scarlet fever, rheumatic infections and diphtheria that pyrexia alone does not affect the vitamin C reserves or utilization. Reduction of the serum level of vitamin A in febrile states has been observed during infection and artificially produced pyrexia (48, 70, 388, 414). Available data indicate, however, that such effects are more likely due to impaired liberation of vitamin A from the liver to the blood than increased utilization. May et al. (292) report a spontaneous rise in the vitamin A level of the blood after fever abatement without any vitamin A being taken; and similar results have been observed for typhus fever (142), rheumatic fever (371), and pneumonia (241). The effects of fever in preventing absorption of vitamin A and A-active carotenoids have previously been reviewed (70, 211). Schaefer (361) has reported an increased iron requirement during the febrile stage of infection in children, but whether a similar effect would occur in artificial pyrexia has not been determined.

Evidence is available that strenuous physical exertion, particularly if continued for prolonged periods of time, will increase body requirements for various nutrients. The primary need is for more calories, a point recognized since antiquity but placed on an exact basis by the work of Atwater and Benedict (21) and others (34, 112, 379). Christensen et al. (67) have found that during caloric restriction

(fasting) pronounced physical exertion may result in considerable deterioration in a period as short as two days. Under such circumstances even a single meal containing only a fraction of the day's requirements will result in significant improvement (148). The marked increase of metabolism during strenuous physical exertion (112) and the consequent ingestion of larger amounts of food both serve to increase body requirements for vitamin B_1 (81). In this regard, Cowgill et al. (84) have pointed out that the length of time required for dogs to develop anorexia on a vitamin B deficient diet was one-third to one-half less in dogs permitted to exercise each day than in those who were not; and Guerrant and Dutcher (179) have similarly observed an increased thiamine requirement subsequent to exercise in the rat. Circumstantial evidence indicates that a similar relationship pertains to man. Thus Egan (119) reports that in an epidemic of beriberi on shipboard, the stokers were affected more seriously than the sailors in the ratio of 34 to 3, an effect that may have been due to the greater physical exertion of the former and the resulting higher food consumption. Similarly in the literature of beriberi, epidemics in Japan, Burma, Siam and elsewhere a great preponderance of beriberi is noted in males. This, again, may be due in part to the harder physical work to which men are customarily subjected. The high incidence of thiamine deficiency observed in the past in mental hospitals among patients with delirium or mania may have been due in part to a greater requirement, resulting from increased psychomotor activity and a corresponding elevated metabolic rate (437, 438). Recently, the subject has been attacked experimentally in man. Egana et al. (120), Johnson et al. (234) and others (18, 24, 438) report that severe physical exertion on a diet deficient in the B vitamins greatly hastened the onset of symptoms. Physical deterioration, according to Johnson et al. (234) occurred within one week and could be prevented or cured by the entire B complex containing 0.6 mgm. of thiamine daily, but not by 2.0 mgm. of thiamine hydrochloride alone, suggesting an increased requirement not only for thiamine hydrochloride but other B complex factors as well. Diametrically opposite results were obtained, however, by Keys et al. (251, 252, 253) who, in the most careful study of the subject so far reported, observed no evidence of physical or psychologic deterioration after fourteen days on a diet containing as little as 0.16 mgm. of thiamine, 0.15 mgm. of riboflavin, and 1.8 mgm. of niacin per 1000 calories. These results, according to Keys (251), "completely negate the claims that important effects on work performance occur in a few days or weeks when normal young men do very hard work while subsisting on a diet severely restricted in the vitamins of the B complex." Similar experiments extending for longer periods of time are necessary, however, to establish the effects of physical activity on thiamine requirements and to determine to what extent strenuous physical exertion will hasten the onset of symptoms on a diet deficient in the B vitamins. Available data indicate that physical exertion does not significantly increase body requirements for vitamin C. Johnson et al. (235) observed no adverse effects in subjects engaged in manual labor on a vitamin C free diet during a period of eight weeks. Similar results were obtained by Crandon et al. (85, 86) and others (342) for even longer periods, in spite of a blood plasma virtually free of ascorbic acid

after the sixth week of feeding. Recent experiments by Darling et al. (101) and others (335) confirm the earlier findings of Chittenden (66) that physical exertion does not increase the protein requirement of man.

Requirements for essential nutrients may be significantly increased during hyperthyroidism both in experimental animals and man. This may occur in exophthalmic goiter, toxic adenoma or malignancy of the thyroid gland or from the administration of thyroxin, desiccated thyroid or iodinated casein. These requirements are due, in part, to an increased metabolism and food consumption; but they also reflect a specific response to the thyroid hormone *per se*. The subject has been extensively reviewed, with particular emphasis on vitamin requirements, in a most thorough manner by Drill (109). An increased requirement for calcium, protein, and vitamins A and C was observed during hyperthyroidism, both in experimental animals and man; but the B vitamins apparently were most affected (109). As early as 1932 Himwich et al. (215) found that the "vitamin B" requirement was increased in the hyperthyroid dog, and similar results were soon obtained for other species (82, 108, 401). In 1938 Drill and co-workers initiated a series of experiments on the effects of vitamin B_1 and yeast on adult hyperthyroid rats that had lost weight. They found that further loss in weight could be prevented by vitamin B_1 alone, but that rats did not regain their initial weight unless a rich source of the vitamin B complex was also supplied (108, 111). Subsequent work has demonstrated that pyridoxine and calcium pantothenate could effectively replace the vitamin B complex in the diet of the hyperthyroid rat (110). Thus, in addition to vitamin B_1 both pyridoxine and pantothenic acid are required in increased amounts during experimental hyperthyroidism in the rat.

B. *Pregnancy and lactation.* Pregnancy and lactation constitute two additional stress factors which may significantly increase body requirements for essential nutrients. In part, these requirements may be due to increased metabolism; they also reflect, however, an increased utilization in the formation of fetal or placental tissue and increased secretion in the form of milk. During the early period of pregnancy the basal metabolic rate is maintained within normal limits. During the later months, however, the basal metabolism rises and at term may reach an average value of plus 30 (280). Some observers believe this increase can be entirely accounted for by the metabolism of the fetus; others maintain, however, it is due in part to factors resident in the maternal organism in the nature perhaps of increased thyroid or pituitary activity (92, 129). During lactation, even higher values are obtained, due most likely to the increased activity of the secreting mammary glands. The increased metabolic rate observed above is correlated with a corresponding increase in thiamine requirement. Toverud (418) found that 46 per cent of a group of 114 pregnant women excreted practically no vitamin B_1 in their urine, and that a daily dose of 4 to 5 mgm. of thiamine was necessary to obtain an excretion corresponding to normal nonpregnant controls. Lockhart et al. (274) observed that three times as much vitamin B_1 were required to produce a urinary excretion peak in pregnant and nursing women as were required in normal controls. In agreement with the

above, numerous reports are available on the increased incidence and severity of polyneuritis during pregnancy and lactation in man and its successful treatment with vitamin B_1 (9, 319, 440). Evans and Burr (137) found that the nursing rat required five times its usual amount of vitamin B. Available data indicate that riboflavin requirements are also increased during pregnancy in man (43, 114).

All the evidence points to an increased requirement for vitamin C in pregnancy and lactation. Blood levels of vitamin C fall progressively throughout pregnancy (61, 123, 385, 407, 411). Elmby and Becker-Christensen (123, 124) found that the serum ascorbic acid level in non-pregnant women was double that of pregnant women of the same social class. Bucher (339) asserts that pregnancy causes a four to six-fold increase in requirements for vitamin C. Other workers are more conservative but agree requirements are increased by at least 100 per cent (149, 322, 418). During lactation, vitamin C is required not only for the needs of the mother but for those of the child, as well. Baumann and Rappolt (28, 29) have calculated that a nursing mother secreting 800 cc. of milk a day, containing at least 5 mgm. of vitamin C per 100 cc., requires a minimum of 50 mgm. of vitamin C beyond the normal daily requirements. Other workers have arrived at substantially the same figure (123, 418, 435). Protein and calcium requirements are also increased during pregnancy and lactation in man (117, 152).

C. *Detoxification.* In recent years considerable work has been published concerning the rôle of amino acids and other nutrients in detoxification. By feeding substances which are "detoxified", deficiencies of glycine, methionine and other nutrients may be produced, correctable by the administration of increased amounts of the appropriate nutrient. If benzoic acid or one of its salts is administered, either parenterally or by mouth, only a part of it is excreted as benzoate, the major portion being converted to hippuric acid, in which form it may be recovered from the urine. Since glycine is considered a dispensable amino acid and can readily be synthesized from other components of the diet, it was not regarded as a nutritive essential and hence a glycine deficiency was not considered probable, at least for the rat. Marked inhibition of growth has been observed, however, in rats fed benzoic acid or its sodium salt on a low protein diet, suggesting that glycine requirements for detoxification were not being met and that animals were suffering from a "glycine deficiency". Removal of benzoic acid or sodium benzoate from the experimental ration or the administration of increased amounts of glycine restored normal growth (175, 433). Inhibition of growth has been observed following administration of bromobenzene to rats, presumably due to a cystine deficiency resulting from the excretion of mercapturic acid (392, 434). Cysteine, methionine and glutathione were all effective in restoring normal growth (392). Growth was similarly retarded following the feeding of biphenyl or chrysene and restored to normal on the addition of methionine or cysteine (431). A deficiency of labile methyl groups has been reported in rats fed diets containing 1 per cent nicotinic acid or its amide. The attendant growth retardation was alleviated by methionine, choline plus homocystine or choline plus cystine, but not by choline, cystine or homocystine alone (195, 393).

Requirements for methionine and other nutrients may be significantly increased following exposure to various drugs, chemicals and other toxic compounds. High protein diets have been shown to exert a beneficial influence when administered to experimental animals exposed to chloroform, carbon tetrachloride or benzene, or when administered to animals given toxic amounts of selenium, trinitrotoluene, arsphenamine or mapharsen (154, 181, 299, 362, 382, 386). Following the demonstration of the protective action of protein, there was an intensive search for the fraction or fractions chiefly responsible for it. Methionine, and to some degree cystine, proved to be most responsible for the above effects. Miller et al. (303) found that methionine, and to a lesser extent, cystine, protected against chloroform poisoning in the protein depleted dog. The beneficial effect of methionine has similarly been observed following administration of mapharsen or exposure to carbon tetrachloride in dogs (155, 307), 1,2-dichloroethane in rats and mice (208, 209) and trinitrotoluene and carbon tetrachloride in man (33, 118). The available data indicate that requirements for methionine and other amino acids may be significantly increased following exposure to various noxious agents. The increased requirements are due to the use of these amino acids for detoxification. On a low protein diet, insufficient amounts of such amino acids may be present to meet the requirements for maintenance plus detoxification with the result that an amino acid deficiency may develop. The effects of such a deficiency will be particularly marked in the liver cell, since the liver is the organ where detoxification primarily occurs. The hepatotoxic effects of chloroform, carbon tetrachloride and other toxic substances may be explained therefore, at least in part, on the basis of a methionine deficiency in the liver cell. Particularly pertinent in this regard is the similarity of symptoms resulting from a dietary deficiency of methionine with that obtained on the administration of noxious substances requiring methionine for detoxification. It appears further that the body will employ nutrients preferentially for detoxification, even at the expense of sacrificing its own tissues to obtain the required material. A recent report by Croft and Peters (87) is pertinent in this regard. It has been demonstrated that the excessive urinary loss of nitrogen following severe burns could be checked both in the human (409) and the rat (87) by the ingestion of large amounts of protein. Croft and Peters found that methionine was equally effective in the rat (87). Such data suggest that following a severe burn, methionine is required in increased amounts, at least by the rat; that insufficient amounts of this amino acid may be available from dietary sources, and that body tissue will be broken down to meet the increased demand. Increased urinary nitrogen is explained on the basis of deaminization of excess amino acids and the resulting rise in urinary nitrogen excretion.

Detoxification may similarly increase body requirements for ascorbic acid. An increased requirement for this vitamin has been observed following the administration of toxic doses of l-tyrosine (368), gold (96, 354, 369), lead (219, 220, 287) and arsenic compounds (59, 75, 76, 77, 95, 96, 397, 423), benzene (185, 270, 300), phosphorus (352), trichlorethylene, T.N.T. (99), barbiturates (167), anesthetics (115, 266), sulphonamides (40, 97, 98), hydrazine (36) and various other

compounds. It appears that ascorbic acid combines with at least some of these substances and is excreted in such combination in the urine. Similar detoxification effects are observed toward diphtheria toxins (254, 375, 376), tuberculosis (231, 349), and many other infectious diseases (331). Guinea pigs which died from diphtheria intoxications showed reduced vitamin C content of the adrenals (196, 243), whereas no significant differences were found between the vitamin C content of the adrenals of animals injected with a sublethal dose of diphtheria toxins and those of normal animals (417). Bacterial toxins can cause a decrease of as much as 50 to 85 per cent of the normal vitamin C content of the adrenals (198, 277, 416).

Requirements for other nutrients may similarly be increased following the administration of various drugs, toxins, poisons and related substances. Higgins (214) found that the toxic effects of orally administered promin (sodium p,p′ -diaminodiphenyl sulfone -N,N′ -didextrose sulfonate) were counteracted by increased amounts of thiamine, riboflavin and pyridoxine. Fishman and Artom (145) observed that the toxic effects of dl-serine were modified by ingestion of pyridoxine. Sandground (355, 357) and Sandground and Hamilton (358, 359) showed that p-aminobenzoic acid was highly effective as a detoxicant for lethal doses of phenyl arsenates such as "Tryparsamide", arsanilic acid, carbarsone and acetarsone; and virtually the same results have been obtained with the pentavalent antimonial compound "Stibosan" (sodium-m-chloro-p-acetylaminophenyl stibonate) (199, 356). P-aminobenzoic acid was found to alleviate the symptoms of hydroquinone poisoning (289) and to reduce, at least in rats, the toxicity of neoarsphenamine (279). More recently, p-aminobenzoic acid has been shown to be of value in the treatment of experimental tsutsugamushi disease (scrub typhus) (316, 386), murine (170, 171, 193) and epidemic typhus (193) and Rocky Mountain spotted fever (17, 192). Clinical trial of p-aminobenzoic acid has demonstrated it to be of value in the treatment of louse-bourne typhus fever (454) and apparently Rocky Mountain spotted fever as well (347). Vitamin K therapy will raise the lowered prothrombin level due to sulphonamide (72), aspirin and salicylate therapy (372), dicumarol (88, 103, 373) or quinine sulfate (334). Iodide will protect against poisoning by thiourea (172) or ANTU (alpha-napthylthiourea (60)). Selenium poisoning was prevented by the administration of arsenic salts (113, 313).

Data are lacking regarding the specific mechanisms through which the above nutrients exert their effects. Some results were apparently due to detoxification, but the *modus operandi* remains obscure; others may reflect a pharmacologic, as distinguished from a nutritional, response. The net result, however, remains the same, namely, that a toxic or deleterious effect was alleviated or prevented by increased amounts of a specific nutrient. It may be added further that these effects occurred in animals that were normal at the start of the experiment and were fed diets that were qualitatively and quantitatively adequate in the absence of the various stress factors employed; and further, that animals responded to doses of nutrients that could be supplied by diet alone.

D. *Anoxia.* Available data suggest an increased vitamin requirement in the

therapy of shock and anoxia. Govier and Greer (160) found that thiamine would prolong the life of dogs in hemorrhage-induced shock, although the animals were in excellent health at the start of the experiment and were fed a diet apparently adequate in all respects. Blood pyruvic acid was determined in a number of animals during shock, and levels as high as 4 to 5 mgm. per 100 cc. were observed in contrast to the normal range of 1 to 2 mgm. per cent (159). This level is actually higher than that seen in most cases of beriberi, clinically. Thus, it would appear either that these animals became thiamine deficient as shock was induced or that their thiamine became incapable of functioning in a normal manner. It is well known that for thiamine to be effective as a coenzyme in pyruvate metabolism, it must be phosphorylated to diphosphothiamine or cocarboxylase. Under normal conditions most of the thiamine is in the phosphorylated form, but under abnormal conditions, such as shock, Govier (157) suggests that breakdown of cocarboxylase may occur, thus reducing the amount of metabolically "active" thiamine. Ochoa (321) has shown in vitro that under anaerobic conditions such breakdown of cocarboxylase does occur, probably by means of a phosphatase. Dephosphorylation of cocarboxylase was similarly observed by Govier and Greig (161) in the tissues of dogs after shock. Govier points out, therefore, that although shocked animals may be well supplied with thiamine, they may nevertheless become in a sense thiamine deficient, since their thiamine becomes converted to a form which is useless in tissue metabolism (157). Under such circumstances large doses of thiamine are required to raise the intracellular concentration of thiamine so that resynthesis of cocarboxylase may occur. A similar breakdown of cocarboxylase has been observed in dogs allowed to breathe oxygen-nitrogen mixtures containing 10 per cent oxygen (172). It appears, therefore, that anemic anoxiaand anoxic anoxia both produce a tissue anaerobiosis which results in a breakdown of cocarboxylase. Cocarboxylase, however, is not the only coenzyme which may be broken down in anoxic conditions nor are shock and anoxic anoxia the only conditions in which coenzymes are broken down. Greig (173) found that in shock a pronounced breakdown of coenzyme I and alloxazine adenine dinucleotide (flavine adenine dinucleotide) may occur and that increased amounts of nicotinic acid and riboflavin are required respectively for re-synthesis to take place. Destruction of coenzyme I has also been observed following experimental coronary ligation (158).

E. *Miscellaneous factors.* A group of factors not readily classified under one heading may also raise body requirements for various nutrients or hasten the onset of deficiency states. Prolonged parenteral administration of glucose, particularly to patients in a borderline state of nutrition, increases body requirements for thiamine and may precipitate deficiency disease (238, 404). Excessive consumption of carbohydrate acts in the same way; beriberi due to this cause has, in fact, been reported (153, 395). Exposure to sunlight or other forms of irritation such as radiant energy, dirt, filth, chemical trauma, tight clothing or repeated friction may precipitate skin lesions in nicotinic acid deficient persons (200, 350, 378). Johnson and Eckhart (232) found that sunlight hastened the onset of corneal vascularization in riboflavin deficient rats, and Sydenstricker

et al. (405) have suggested that exposure to bright light might cause destruction of riboflavin in the cornea with resulting ocular signs in the absence of lesions in other tissues. Mills et al. (308) observed that rats on a vitamin K deficient diet had four times as much internal hemorrhage when kept at 90 to 91 degrees F. as rats kept at 68 degrees F., an effect presumably due to an increased requirement for vitamin K at the higher temperature. For the water-soluble vitamins, however, requirements not only were not increased at higher environmental temperatures but in some cases were actually lower, due to decreased activity and reduced food intake (207, 255). Riboflavin requirements may be increased at high altitudes and other conditions where the oxygen supply is decreased (310). Administration of growth hormone has accentuated deficiency symptoms in the vitamin A deficient rat (133). An increased requirement for folic acid was reported for rats fed reduced caloric intakes (132). A pyridoxine deficiency was observed in the suckling young of rats fed increased amounts of thiamine during pregnancy and lactation (340).

Evidence is accumulating that in adition to the major nutrients, substances are present in our diet which may be required in increased quantities during conditions of stress. Such factors are apparently dispensable under normal conditions or their requirements are so small that they may readily be met by amounts present in the diet or through the synthetic activity of the intestinal flora or the animals' own tissue. Certain drugs or other stress factors may, however, increase requirements for these substances to the extent that deficiencies occur, manifest by retarded growth or tissue pathology, and preventable by the administration in appropriate amounts of the missing nutrient. Many effects commonly attributed to drug toxicity or endocrine hyperfunction may in reality reflect a tissue deficiency due to an increased requirement for one of these "minor vitamins". A recent report by Ershoff and Hershberg (135) is a case in point. It has been demonstrated that requirements for thiamine, pyridoxine and pantothenic acid are increased in the hyperthyroid rat (110). . The above authors found that still another factor, present in yeast, is required in increased amounts by the hyperthyroid rat. Length of survival was significantly longer in thyroid-fed rats on yeast containing rations than those on similar diets containing synthetic B vitamins (135). Furthermore, neither wheat germ, pancreas, nor individual supplements of thiamine, riboflavin, pyridoxine, calcium pantothenate, nicotinic acid, p-aminobenzoic acid, inositol, folic acid nor biotin prolonged the life of thyroid-fed rats on synthetic rations; liver, however, was as effective as yeast in this regard (131). Moreover, it contains still another factor required in increased amounts by the hyperthyroid rat. It is well known that excessive thyroid feeding retards the growth of young rats. Futhermore, if feeding is begun at weaning, ovaries remain infantile, both in weight and microscopic appearance (130). Both effects, i.e., retardation of growth and failure of ovarian development, were counteracted by liver, although yeast, wheat germ, pancreas, thiamine, riboflavin, pyridoxine, calcium pantothenate, nicotinic acid, p-aminobenzoic acid, inositol, folic acid and biotin were ineffective in this regard (131). Similar experiments have been reported by Ershoff and Deuel (134) in which

alpha-estradiol served as the stress factor. Male and female rats were raised to maturity on purified rations containing excessive amounts of alpha-estradiol. Two basal rations were employed: in one, the B vitamins were administered as synthetic factors; in the other, they were present as yeast. After sixty days of feeding, both body and gonadal weight were significantly greater in the yeast series. The list of minor vitamins may be further extended to include substances effective as anti-hypertensive agents. Harrison et al. (202) prepared kidney extracts which were effective orally or parenterally in lowering the blood pressure of rats rendered hypertensive by surgery or renin, although without effect in normal animals. Similar results were obtained with marine oil concentrates, both in experimental animals (177, 427) and man (176, 329). At first, such results were attributed to the vitamin A content of the preparations employed; subsequent work has demonstrated, however, that the blood pressure-reducing factor was some unrecognized substance and that vitamin A was neither the antipressor substance nor its precursor (177, 312). More recently, Grollman (176) obtained the active principle in aqueous solution and suggested it might be identical or of related chemical structure to the active substance in renal extracts.

SUMMARY

Malnutrition may be caused by conditions other than a primary dietary deficiency. These include factors that interfere with the absorption and utilization of nutrients or those that increase their destruction and excretion. Body requirements for essential nutrients may furthermore be significantly increased for purposes of detoxification or by factors such as physical exertion, fever, drugs, toxins, abnormal environmental conditions, pregnancy, lactation, hyperthyroidism and related conditions which result in an increased metabolic requirement on the part of the tissue cell. The net result of such factors is an increased body requirement beyond the usual or average range and the precipitation of nutritional deficiencies on diets that would otherwise be adequate were such factors not operative. In the present review a critical survey has been made of current literature in respect to these factors and their effect on the vitamin, mineral and amino acid requirements of experimental animals and man.

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